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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this research is to develop methods of generating tumor specific small peptides that will bind to human cancers. The scope of this research is to construct peptide-displayed random peptide libraries as a source of peptide ligands that will target human cancers. An in vivo selection process will select ligands present in the library. Preclinical toxicity testing in a murine model is in the scope of this project and much of this work has been completed. The preclinical work in mice strongly supports the use of this technology in humans because the toxicity levels appear very low and because panning experiments have resulted in enrichment of peptide-phage clones that are likely tumor-specific.

Phase I testing of detecting ligands in human cancer patients is within the scope of this research. Our preliminary data has led to approval by the FDA to proceed with human clinical study. We are prepared in this third year of grant support to safely enter patients into this clinical study. We will continue to develop methods related to evaluation of clones obtained from in vivo panning. We remain highly optimistic that peptides with significant affinity to tumors in breast cancer patients can be obtained.

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ABBREVIATIONS:
RPL- random peptide library
FDA- United States Food and Drug Administration
HB- homogenization buffer
TU- transducing unit
IHC- immunohistochemistry
HRP-horse radish peroxidase
ϕ-phage
LAL= Limulus Amebocyte Lysate

INTRODUCTION:

This report describes the second year of research designed to investigate the effectiveness of *in vivo* screening of phage-displayed random peptide libraries (RPLs) in cancer patients. The purpose of this research is to identify small peptides that bind specifically to breast tumor targets, which can ultimately be used to develop effective cancer therapeutics with high specificity for tumor cells and low toxicity to normal cells. Peptides identified by phage-display RPL technology cannot only bind targets with high specificity, their small size is more optimal for drug development than larger tumor-binding molecules such as antibodies. We have completed preliminary toxicity testing of intravenous delivery of RPLs to mice and found them to be relatively nontoxic. Panning experiments in these same animals have demonstrated that enrichment occurs and interesting consensus sequences have been identified. The FDA has approved our protocol for use in humans. Approval by the University of Vermont Committee on Human Research in the Medical Sciences (CHRMS) is complete and the final approval by the Surgeon General's Human Subjects Research Review Board (HSRRB) is nearly complete. We are now fully prepared in this third year to accrue patients into the study

BODY:

Task I. Construct a large panel of random peptide libraries (months 1-14) For each system, five libraries will be constructed with disulfide-constrained loops ranging in size from 8-12 amino acids. Each system displays systems in a different structural and/or spatial context.

Three new peptide libraries have been constructed this year. All three libraries were constructed in a pVIII phage display system. Random peptide libraries were created by cloning synthesized random DNA oligos into the N-terminal coding region of gene VIII. The inserts containing random sequences were expressed as peptides on the N-terminus of the gene VIII major coat protein, each phage displaying several thousand copies of the major coat protein per phage particle and only a small peptide can be displayed from each copy with phage viability maintained. Therefore, most gene VIII systems use a phagemid system that allows display of a controlled number of fusion VIII proteins in combination with wild-type major coat protein

supplied by helper phage. The potential advantage of this gene VIII system is that it allows a different spatial presentation of peptides, farther apart from each other than on gene III systems, and present at a much greater copy number, typically several hundred per phage particle. Other advantages are that the displayed peptide is fixed in position on the outer coat of the phage minimizing possible interactions of the displayed peptides with each other. A potential disadvantage is that low affinity ligands may be selected. This is due to avidity effects. If a phage has only a few copies of displayed peptide there are limited opportunities for the entire phage particle to attach to a target molecule. If the phage does attach it is because the low copy number of peptides has sufficient binding affinity to keep the phage attached. If the phage has many copies of displayed peptides several attachment points are present. The peptide may have low affinity for a target but since there are so many peptides present the phage may hold strongly to the target and get selected.

Specifically, we have constructed three pVIII peptide libraries, two display 18 random amino acids and one displays 19 random amino acids. Two cysteine residues were designed in these three libraries to display a constrained ten or eleven-residue loop (refer to table below for design information on each library). Peptides that are conformationally constrained often possess higher affinity for a target than their linear counterpart [1]. In previous work in our laboratory, we identified a peptide that needed to be in a cyclic conformation in order to bind to the SH2 domain of Grb2 [2]. The libraries were designed so X = any amino acid encoded by NNK, where N = G, A, T, C and K= G or T. The NNK cloning scheme, a method commonly used in phage display, eliminates the potential for two stop codons and still encodes all twenty amino acids. The four amino acids on the N- and C-termini of the loop were included to add a linear component to the cyclic peptide, which might allow the peptide to anchor itself with greater affinity onto the target [1, 3].

The three random pVIII peptide libraries were created in p8V5 system using cloning methods described by Affymax. This method converts two annealed oligos, one containing the random NNK sequence, to fully double stranded DNA with Sequenase T7 DNA polymerase. The oligo was then digested with BSTXI and BsiHKAI, ligated in the BstXI cloning site of p8V5 phagemid vector, and transformed into MC1061F' cells. The VCSM13 helper phage was used to generate phage particles from these phagemid libraries. The library complexity was determined by the original number of transformants. DNA sequencing of the N-terminal region of gene VIII of randomly chosen phage clones confirmed the presence of random inserts.

Date	Library Name	Library Design	Complexity
10/25/00	pVIII-10mer with arms (#p8Lib-102500)	X_4 C X_{10} C X_4	6.2×10^7TU
11/07/00*	pVIII-10mer with arms (#p8Lib-110700)	X_4 C X_{10} C X_4	9.02×10^9TU
12/06/00	pVIII-11 mer with arms (#p8Lib-120600)	X_4 C X_{11} C X_4	7.74×10^8TU

*Note: The pVIII-10mer library was made a second time to increase the complexity of the library.

Task II. Establish the safety of intravenous administration of phage RPLs.

Mice were chosen as the preclinical model for toxicity evaluation. The choice of species, species strain, and several aspects of the protocol design were done in an active consultation with the Food and Drug Administration. The results demonstrated that minimal to no apparent toxicity was encountered following administration of the phage-displayed RPL library under a variety of administration schemes. In addition, panning experiments demonstrated enrichment of phage harvested from excised tumor nodules (see below Task III). The enriched phage had multiple consensus sequences. Demonstration of 1) minimal toxicity and 2) meaningful enrichment of phage collected from harvested tumors resulted in approval by the Food and Drug Administration (FDA) to proceed with human clinical studies (IND # BB-IND#9145).

METHODS:

The library utilized for the mouse toxicity experiments contained a variable 9-amino acid peptide flanked by cysteine residues inserted into the Gene III protein of the phage fUSE5[6, 17]. The phage were quantified by titering essentially as in G. Smith's protocol "Cloning in fUSE Vectors" Feb 10, 1992. K91 Kan *E.coli* were grown in 10 ml Terrific Broth supplemented with kanamycin (KAN) to mid log phase (O.D. 1.0 at 600nm) with vigorous shaking at 37°C. Sheared pili were allowed to regenerate with gentle shaking for 10 minutes. The culture was placed on ice for 10-60 minutes. Dilutions of phage were prepared in phosphate buffered saline (PBS) and 10 µl aliquots were pipetted into disposable culture tubes. To the droplet, 10 µl of K91 culture were added and incubated for 10 minutes at room temperature, followed by the addition of 1 ml LB (Luria-Bertani) with 0.2 µg/ml tetracycline and incubation at 37°C with vigorous shaking for 25 minutes. Samples were placed on ice for 10 minutes prior to plating 20 µl of infected cells onto LB plates supplemented with tetracycline. The strain of filamentous phage used in the animal studies is fd-tet, which allows infected bacteria to grow in the presence of tetracycline. Plates were incubated overnight at 37°C. Results were expressed as transforming units (TU).

Preparation of RPL for injection: Filamentous peptide-phage from a previously constructed library were prepared from *E. coli* cultures grown overnight on 2xYT media agar plates supplemented with kanamycin and tetracycline. The phage particles were resuspended in phosphate buffered saline with prokaryotic protease inhibitors (PBS-PPI) by "sweeping" the agar with an angled glass rod. The phage suspension was centrifuged twice to remove bacterial cells and filtered with a 0.22 µm polyethersulfone membrane (PES) to completely remove any remaining *E. coli* cells. The phage were concentrated by precipitation with 0.15 ml polyethylene glycol (PEG)/ml of filtrate. The resulting pellet was resuspended in fresh PBS-PPI and filtered through a pyrogen-free 0.2 µm cellulose acetate filter.

Endotoxin removal and testing: Endotoxins were removed from the preparation by performing three 1% (v/v) Triton X-114 extractions. The phage were concentrated with PEG again and the resulting pellet was resuspended in PBS-PPI. The phage suspension was shaken 10 min at 200 rpm on ice, followed by centrifugation. The supernatant containing the peptide-phage was passed through a pyrogen-free 0.2 µm cellulose acetate filter to sterilize the preparation. The Limulus Amebocyte Lysate (LAL) gel clot assay (Charles River Endosafe) was used to determine the level of endotoxins remaining in the preparation and for potentially interfering substances in the preparation that might inhibit the gel clot reaction.

Sterility testing: This was performed by inoculation of the product into Fluid Thioglycollate Media and Tryptic Soy Broth. Sterility testing was performed as described in the Code of Federal Regulations (21CFR610.12) on representative preparations.

Description of mice: Three strains of mice (Jackson Labs in Bar Harbor, Maine) were used for the toxicity studies: FVB, BalbC, and MRL/MpJ-fas$_{LPR}$(MRL). The FVB and BalbC mice are normal strains. The MRL mice develop massive lymph node enlargement, or lymphoproliferative disease, beginning around 8 weeks of age. The MRL mice were chosen for in vivo screening because the lymph nodes become markedly enlarged and form multiple palpable tumors. MRL mice have the disadvantage of dying rather early and somewhat unpredictably compared to other strains of mice.

Survival Surgery Protocol: All animal procedures were approved by the Institutional Animal Care and Use Committee. The mouse was weighed and positioned on a warming pad to maintain body temperature. The mouse was anesthetized with halothane. The eyes had ophthalmic ointment applied and were protected from bright light. Breathing pattern and toe pinch was used to monitor level of anesthesia. A warm compress was applied to dilate the tail vein. Through a 29 gauge needle, 250 µl or less of sterile peptide-phage preparation was injected into the tail vein. The material was allowed to circulate for 10 minutes. Electric clippers were used to shave the area immediately surrounding the tumor to be excised. Isopropyl alcohol was used to cleanse the operative field and sterile drapes and instruments were used. Ten minutes following injection, the subcutaneous tumor of interest was excised through a small skin incision. The incision was closed using interrupted 5-0 nylon suture. Mice were injected subcutaneously with ½ dose of buprenorphine (0.05 mg/kg) for pain and the remaining ½ dose at 12 hours post surgery.

Collection of phage from harvested tumors: The tumor was rinsed with PBS-EPI (eukaryotic protease inhibitors), weighed, minced and homogenized in homogenization buffer (HB) (RPMI supplemented with 1.8 µg/ml insulin, 2mM L-glutamine, and 10% calf bovine serum). The homogenate was centrifuged and rinsed several times with HB to eliminate unbound phage, and the final pellet resuspended in HB. An excess of ready cells (described earlier) were added to rescue the remaining tissue-bound phage. The suspension was incubated with gently shaking for 1 hour at 37°C, followed by the addition of tetracycline (0.2 µg/ml) and a 25-minute incubation with vigorous shaking at 37°C. The suspension was centrifuged and the supernatant containing peptide-phage removed for quantitation and amplification. An aliquot was saved for titering while the remainder of the rescued phage were plated on 2xYT agar plates, supplemented with kanamycin/tetracycline, and amplified overnight. Amplified peptide-phage (□amp1x) were subsequently harvested and purified for injection as described earlier (Amplification of RPL for injection).

Phage Titering Protocol for Harvested Organs: Tissues (10-100 mg) for titering were weighed, homogenized with disposable pestles in a small amount of (PBS-EPI), and incubated with an equal volume of ready cells for 1 hr at ambient temperature. Tetracycline (0.2 µg/ml) was added to the suspension followed by vigorous shaking for 25 minutes at 37°C. The suspension was concentrated by centrifugation (6600 x g, 5 min at 4°C), resuspended in approximately 50 µl PBS-EPI and plated on LB Kan/Tet plates. Plates were incubated overnight at 37°C. One colony=one transducing unit (TU). In some cases, heparinized blood (20µL) was also titered essentially as described above, albeit without homogenization.

Immunohistochemistry Protocol for Mouse Tissues: Tissues for immunohistochemistry (IHC) were fixed in buffered formalin, embedded in paraffin, sectioned

and mounted onto slides. The slides were dewaxed, rehydrated, and treated with Target Retrieval Solution (Dako) at 95°C for 15 minutes. Slides were cooled and washed with TBS for 5 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2/methanol for 15 minutes at ambient temperature, followed by Protein Serum Block (Dako) for an additional 15 minutes, and a 5-minute TBS wash. Rabbit α-M13 (Sigma) 7.3 x 10^{-4}mg/ml was added and incubated for 30 minutes, followed by a 5-minute TBS wash. Labeled Polymer HRP, anti-rabbit (Dako) was applied and incubated for 30 minutes, washed with TBS, and followed by DAB+chromogen (Dako) for 7 minutes. Tissues were washed with water, counterstained in Mayer's hematoxylin for 2 minutes, dehydrated, and mounted with coverslips.

Toxicity endpoint: Animals were observed for signs of toxicity by daily monitoring of behavior (posture and activity level), gross appearance (coat), and body weight.

Tissue samples: Ten organs were harvested from each mouse and subjected to three analyses: hematoxylin & eosin staining (H&E) to assess pathology; immunohistochemistry (IHC) to look for the presence of phage particles, and phage titering to determine the number of infective phage remaining. Immediately following euthanasia, samples from ten organs (brain, diaphragm, heart, kidney, lung, bone marrow, lymph node, spleen, gonads, and liver) were harvested, placed in buffered formalin, and were processed for H&E staining and IHC staining. The project pathologist subsequently read all slides.

RPL administration sequence:

Group I- Single Injection of naïve phage: Eight mice (4 FVBs, 4 BalbCs) were administered a single dose of naïve library phage. Two additional control mice (FVB) were administered saline. Two mice of each strain and one control were euthanized for organ harvest at 3 days (to assess acute toxicity). The remaining 2 mice of each strain and one control were euthanized for organ harvest at 3 weeks (to assess chronic toxicity). Blood and the sampled organs were processed for phage titering.

Group II- Single Injection of amplified phage:
A group of 7 mice (3 FVB, 4 MRL) were injected via the tail vein with 6.4 x 10^8 TU φAmp1x in a volume of 250 µl PBS. φAmp1x was peptide-phage amplified from tumor 1 excised from survival surgery mouse (from group 4). A second group of 8 mice (4FVB, 4 MRL) were injected via the tail vein with 8.2 x 10^{10} TU φAmp2x in a volume of 245 µl PBS. φAmp2x = peptide-phage amplified from tumor 2 excised from a survival surgery mouse (from group 4). Mice were monitored daily for toxicity after peptide-phage injection until organ harvesting. During the monitoring period, mice were weighed and observed for signs of toxicity. Two mice of each strain were euthanized for organ harvest at 3 days (to assess acute toxicity) or 3 weeks (to assess chronic toxicity).

Group III- Single injection of naïve phage and serial injection of amplified phage: Six mice (MRL/MpJ-fasLPR) were injected via the tail vein with 3.8 x 10^9 TU naïve peptide-phage(in 250µl). At 48 hour intervals the mice were additionally injected with 3.6 x 10^9 TU ϕAmp1x (in 250µl)and then 2.8 x 10^9 TU φAmp 2x in a volume of 200 µl. φAmp1x and φAmp2x were peptide-phage amplified from tumor 1 and tumor 2 excised from a survival surgery mouse (from group 4). Mice were monitored daily after peptide-phage injection until organ harvesting. Blood was drawn 4 days following injection, and then twice a week thereafter, until blood was shown to be clear of infective phage by titering. The mice were euthanized at 3 weeks and organs harvested for histologic analysis and phage titering.

Group IV- In vivo serial panning: Three MRL mice each bearing at least 3 palpable tumors were evaluated. Naïve RPL was injected and 10 minutes later a tumor nodule was excised. The animals were allowed to recover. Phage were recovered from the tumor, amplified and labeled as "φ Amp1x." One to two days later the mouse was injected with φ Amp1x which had been amplifed from its own tumor. After 10 minutes a second tumor was excised and the mouse was again allowed to recover. Phage recovered from tumor 2 was labeled as "φ Amp2x." One to two days later the mouse was injected with φ Amp2x recovered from its own tumor. Ten minutes after injection a third tumor was excised, the incision sutured and the animal allowed to recover. The phage were eluted from the third tumor and amplified for DNA sequencing analysis. Mice were monitored for signs of toxicity. Mice were euthanized three weeks following the third phage injection/surgery. Immediately following euthanasia organs were harvested for histologic analysis.

Evaluation of immune response in mice to intravenous library phage:

Infusion of library and collection of blood specimens:

Balb C mice (n=2) used for study. On day 1, blood was drawn from the right saphenous vein, incubated for 30 min at room T, centrifuged at 9000 rpm in a microfuge and the serum collected. Serum was stored at –20°C until analysis. Mice were injected IV with approximately 200 µl of PIII Naïve Library (4.9×10^9 TU/ml). On day 3, mice were injected a second time with the same library. On day 6, mouse #1 was unable to be injected a third time. Mouse #2 was injected for the third and final time with the same library.

At 1, 2, and 3 weeks following the third injection, a small amount (~50µl) of blood was collected and processed as above.

Method for Mouse IgG ELISA: On day 1, 96-well plates (NUNC MaxiSorb) were coated with library phage (1×10^7TU's /well) and incubated overnight at 4°C. On day 2, the plated was washed 5x with 0.1% (v/v) Tween TBS (TTBS). This was then blocked with casein for 2 hrs at ambient temperature. Samples of the following were separately added to wells: mouse serum diluted in PBS 1:1000, rabbit IgG or rabbit anti-M13 IgG diluted 1:10,000 in casein. Samples were incubated for 2 hours at ambient temperature and then washed 5x with TTBS. The following were then added to the different samples: goat anti-mouse IgG HRP (diluted 1:4000 in casein) to PBS and serum-treated wells and donkey anti-rabbit IgG HRP (diluted 1:2000 in casein) to PBS, rabbit IgG and rabbit anti-M13 IgG-treated wells. These were incubated for 2 hours at ambient temperature and washed 5x with TTBS. ABTS reagent (Sigma), prepared according to the manufacturer, was then added to each sample and incubated at ambient temperature in the dark. Absorbance was measured at 405nm at 10, 30, and 60 minutes.

RESULTS:

Survival:

All mice entered into this study except two lived to the end of the study period. The first mouse died prior to injection of any phage while in the restraint used for injection. The second mouse died under anesthesia during the second tumor surgery. This appeared to be due to excess administration of halothane anesthesia. The mouse up to that time behaved and appeared normal.

Weight:

The mice in group 2 injected with φAmp1x dropped an average of 9.1% of their body weight on day 1 following injection but their weights returned to baseline by day 2. In group 4 mice, minor weight loss occurred the day after surgery but returned to normal the following day. The weights of all other mice remained stable relative to control mice.

Activity, behavior and appearance:

During the first day after surgery (group 4) mice were less active but this returned to normal by the next day. All other mice had normal activity, behavior and appearance throughout the study.

Histological results of 10 organs per mouse:

Histological analyses of 320 organs from 32 mice injected with phage (this does not include control mice injected with saline) were performed. In group 2 mice, 3 FVB mice had hepatic inflammation in the liver and 1 FVB mouse had lymphoid aggregates in the liver. Sections of liver from mice with hepatic inflammation were subsequently stained with Steiner Stain to rule out Helicobacter or Clostridium infection. No bacteria were identified. All other organs in all other mice appeared normal for that species.

IHC staining for phage particles:

Group 1: At 3 days most tissues were negative. The spleens from all phage-injected mice (n=4) showed trace to 1+ staining in the germinal centers of the lymphocytes. At 3 weeks post phage injection all organs were immunoreactive negative.

Group 2: All tissues were negative for phage staining (IHC) three days following injection of φAmp1x. Most tissues from mice injected with φAmp2x were negative at three days with the exceptions of: 3 livers, 2 spleens, 1 lymph node, and 1 kidney. All tissues three weeks following phage injection were negative for phage particles except for the liver of mouse #16, and a lymph node of mouse #15, both from the φAmp2x-injected group.

Group 3: All tissues were negative for phage three weeks following phage injection.

Group 4: Only one mouse was available for end-experiment IHC and all tissues were negative for immunoreactivity in mouse #2. This is not altogether unexpected as titering is a very sensitive technique for detection of phage, and can detect as little as 1 phage TU. In addition, far less tissue is used in the IHC technique than in titering. Far more phage particles need to be present for a positive IHC signal. It is possible that all the phage we saw by titering in this animal were present in tissues components such as blood, that were washed away during slide preparation for IHC.

Titering of phage from organs and blood:

Group 1: All negative at 3 weeks.

Group 2: Three days after phage injection, there were infective phage present in all of the tissues except for the blood and liver of mouse #3 and the spleen of mouse #2. These mice were injected with φAmp1x. No infective phage were detected in any of the tissues collected three weeks after injection of either φAmp1x or φAmp2x.

Group 3: Blood was free of infective phage 11 days after the third and final injection of peptide-phage. No infective phage were detected in any of the tissues collected three weeks after the third injection of phage.

Group 3: One mouse was available for three-week titering and most tissues were positive.

Endotoxin test results:

Using a LAL reagent with a sensitivity of 0.25 EU/ml, initial preparations contained roughly 10^5 times more endotoxin than is permissible for IV administration in humans. Using the Triton X-114 extractions described above, the amount of endotoxins in our phage preparations decreased to FDA permissible levels. It was also determined that our reagents did not interfere with the endotoxin assay.

Mouse immune response to library phage:

Serum was evaluated for IgG levels in a mouse preinfusion and another mouse three weeks following three injections of phage. A considerable increase in IgG was identified. See figure 1. In order to better define the timing of the antibody response related to infusion of phage, additional studies were done. Blood samples were collected from two mice before and 1, 2, and 3 weeks following 2-3 intravenous injections of library phage administered over 6 days. Serum IgG levels, determined by ELISA, showed a marked increase over baseline (pre-phage) levels 1 week following the final injection of phage. See figure 2.

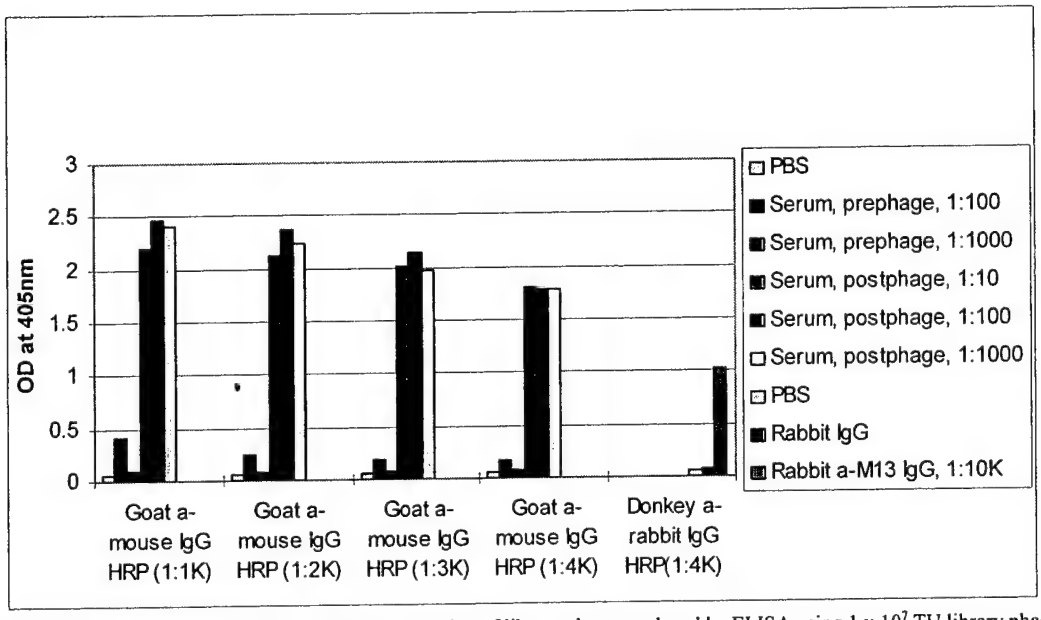

Figure 1. Mouse serum IgG following IV administration of library phage, analyzed by ELISA using 1 x 10^7 TU library phage as target. Note that pre and post phage sera are from two different mice. Mouse dosed with phage was given 3 doses over 5 days: 3.8 x 10^9 TU, 3.6 x 10^9 TU and 2.8 x 10^9 TU respectively.

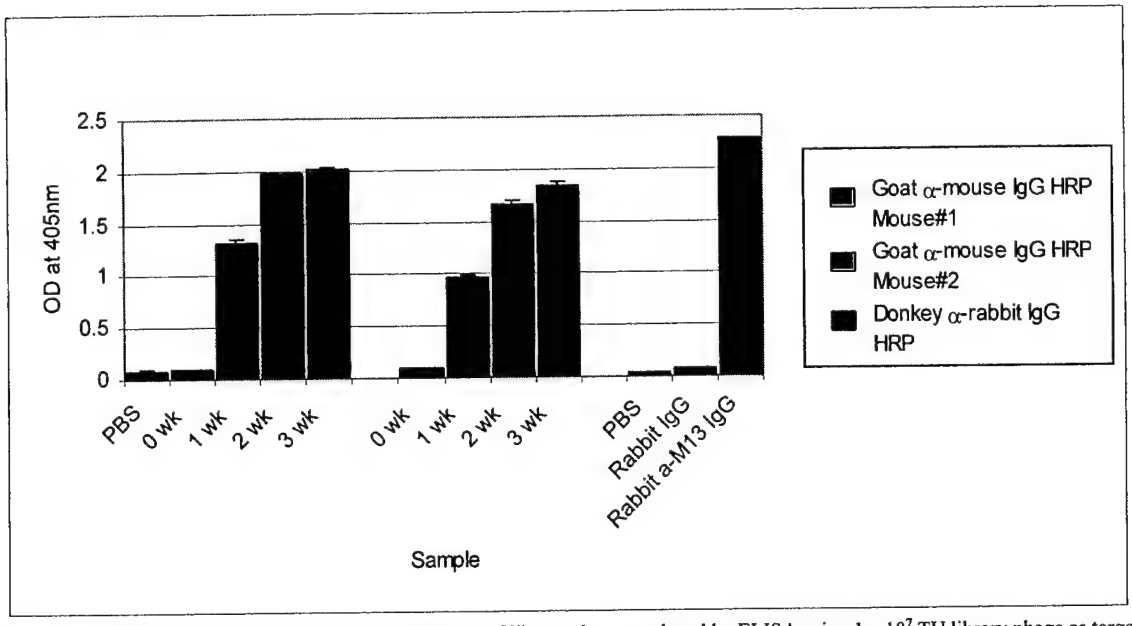

Figure 2. Mouse serum IgG following IV administration of library phage, analyzed by ELISA using 1 x 10^7 TU library phage as target. Mouse#1 was given 2 doses of phage (9.4 x 10^8TU/dose) over 3 days. Mouse#2 was given 3 doses (9.4 x 10^8 TU/dose) over 6 days.

Human IgG immune response assay:

In preparation for our clinical trial, an ELISA was developed to evaluate human IgG levels. Over the course of several assays, we determined the correct dilution for the antibodies, goat anti-human IgG HRP and donkey anti-rabbit IgG HRP. Using the correct antibody dilutions, another ELISA was performed using human serum from a normal volunteer (without exposure to a phage library) as a negative control. This sample was assayed at varying dilutions and compared to a PBS control and purified human IgG at 1, 10, and 100µg (See figure 3). From this assay we determined that human serum diluted 1:1000 gave a response equivalent to PBS. Additionally, we demonstrated that the phage library applied to the ELISA plate as a target was detectable by Rabbit anti-M13 IgG after incubation with donkey anti-rabbit IgG HRP, a control we routinely employ to verify our phage have bound to the microplate.

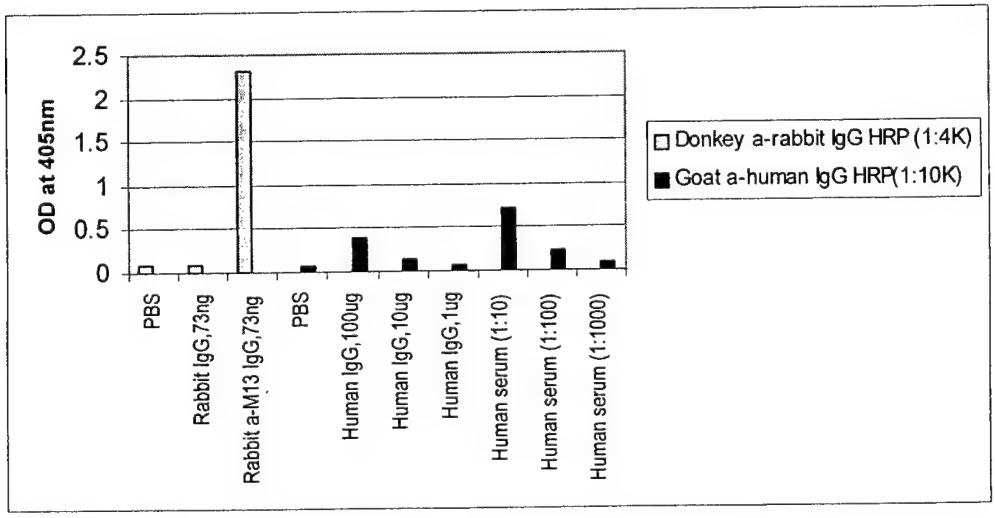

Figure 3. Human IgG determined by ELISA, using 1 x 10^7 TU PIII library phage as target, 60 minutes post ABTS.

Task III. Identify specific tumor-binding phage by in vivo screening and characterize clones.

The preclinical mouse study has provided important information on two goals. The first was definition of toxicity from systemically administered phage-displayed RPL (see Task II above). The second was identification of tumor-binding phage by in vivo screening and characterization of clones. Much of the second goal was presented in the first annual report. This data has been refined and is being submitted as a manuscript from which key elements are now presented.

Consensus amino acid sequences were identified by in vivo RPL screening in MRL mice with tumors. Peptide-phage clones eluted from the third tumor excised from the Group 4 mice were subjected to DNA sequence analysis (20-70 clones per mouse) to deduce the amino acid sequence of the displayed peptides. Several amino acid sequence consensus patterns emerged, several of which are depicted in Figure 4.

Figure 4.

Consensus A:	Clone:
C G S A Y R S P G A C	IV092499-01
C G S A Y R S P G A C	IV092499-02
C G F M S A V P G P C	IV092499-03
C G A F R F L V K D C	IV092499-04
C G D A L P L V N F C	IV092499-05
C D S G G L P L A S C	IV092499-06
C D S G G L P L A S C	IV092499-69
C S Y L P D R S R F C	IV092499-07
C S Y L P D R S R F C	IV092499-56
C S Y L P D R S R F C	IV092499-64
C V S Y S M P P A L C	IV020200-03
C V S Y S M P P A L C	IV020400-54
C G M V S M S P L S C	IV092499-08
C Y H M V S L E N G C	IV092499-09
C V M T S F P W M R C	IV092499-10

Consensus B:	
C E N F V G R N V E C	IV092499-11
C E N F V G R N V E C	IV092499-12
C N M L S L S I P G C	IV092499-39
C N M K V W A T G K C	IV092499-14

C R D L V W R P Q **A C**	**IV092499-13**
C R D L V W R P Q **A C**	**IV092499-42**

Consensus C:

C S L **W R H W** P Y I C	**IV080599-15**[1]
C S L **W R H W** P Y I C	**IV080599-16**
C W R H W V S N Y D C	**IV092499-15**
C T G **H W G I G** E N C	**IV092499-16**
C T T **H W G F** T L C	Koivunen[2]
C S L **H W G F** W W C	Koivunen
C R R **H W G F** E F C	Koivunen

Consensus D:

C S H P S **M S R G** S **C**	**IV020400-14**
C S I S E **M S R G** A **C**	**IV020400-44**

[1]In vivo screening performed by serial pans in three different MRL mice.
[2] Sequences previously reported by panning PRPL to purified MMPs [4]

Figure 4. Consensus amino acid sequences of peptide-phage isolated from tumor tissue after three in vivo RPL screenings. Amino acids that appear at least two times in vertical alignments are bolded and underlined. Some amino acids that appear underlined are not the same but similar. Although the end cysteines were constant in all peptides and homology may not be as significant as the amino acids within the loop, they are still underlined when they line up in the consensus sequence to emphasize which peptides fall into an identical register with respect to the disulfide loop.

Characterization of potential MMP binding clones:

Of particular interest, one consensus pattern, and one peptide in particular, had strong homology with a peptide previously shown to bind to and inhibit matrixmetalloproteinases (MMPs) 2 and 9, molecules that are strongly associated with the metastatic phenotype and are promising tumor targets[4] (Figure 4). In the report by Koivunen, a peptide motif of HWGF was identified by panning against purified MMPs which bound with reasonable affinity to MMPs. Our in vivo mouse tumor panning experiments revealed a nearly identical motif, HWGI. We have been interested in pursuing this peptide motif to better understand possible ligands in a mouse tumor. Further investigation has also been important because it may serve as a positive control that will aid in development of methods to analyze other peptide ligands obtained from in vivo panning.

Immunohistochemistry evaluation of our clones (IV092499-09=CYHMVSLENGC and IV092499-20=CVLSDYIGGSC) on histologic slides of snap frozen mouse tumor did not reveal obvious binding when compared to slides of mouse brain tissue.

We then constructed peptide-phage clones to express as a fusion protein the sequences (MMP2 binder=CTTHWGFTLC and MMP9 binder=CRRHWGFEFC) that were reported by Koivunen to bind to MMP. The purpose was to obtain a positive control and to compare our clones which have similar sequences.

Preparation of MMP fusion phage: Oligos, prepared by BioSynthesis, were annealed and inserted into purified fuseV vector that had been cut with sfi1. The ligated products were then transformed into MC1061F' electrocompetent cells and grown overnight on Luria-Bertani (LB) plates supplemented with tetracycline (TET). Isolated colonies from each clone were subsequently grown in liquid culture, the dsDNA isolated using Qiagen kits and submitted with the fuse V sequencing primer (CCCTCATAGTTAGCGTAACG) to our DNA sequencing facility for confirmation of the correct sequences.

Perform MMP ELISA #1(11.16.00): MMP9 (5μg/ml) was prepared in 50mM Tris pH 7.5, 10mM $CaCl_2$, 150mM NaCl (TCN). On day 1, 500ng of target material (MMP9 or BSA) was added to the wells of a 96-well microtiter plate (Nunc MaxiSorp) and incubated overnight at 4°C. On day 2, the wells were blocked for 2 hours with 1% (w/v) casein in TBS, pH 7.4 (Pierce, Rockford IL). MMP 2-binding or MMP 9-binding phage, blocked for 30 minutes just prior to use with an equal volume of casein, were added (1×10^8 TU/well) and incubated for 2 hours at room temperature. The plate was washed 5x with 10mM Tris, 0.15M NaCl, pH 7.5 containing 0.1% (v/v) Tween 20 (TTBS), followed by sheep anti-M13 HRP (Amersham) diluted 1:1000 in PBS for 2 hours at ambient temperature. The plate was washed 5x with TTBS, ABTS (Sigma) added according to the manufacturer, and the colorimetric reaction read on a microplate reader (Bio-Tek) at 10, 30, and 60 minutes.

Initial ELISA data demonstrated that MMP2 fusion phage clone bound to MMP9 2.2x higher than BSA control and the MMP9 peptide-phage clone bound to MMP9 2.6x higher than BSA control. This level of binding was lower than that expected and was not enough for either clone to be useful as a positive control.

A variety of variables have been evaluated to increase the apparent binding of fusion phage clones MMP2 and MMP9 to purified MMP9 by ELISA. This included 1) increasing the number of phage/well, 2) using purified MMP2 (in addition to MMP9) as target, 3) assay with fresh phage instead of after titering, 4) adding negative control clones, 5) adding one of our MMP-similar clones (Clone - IV092499-16; sequence- CTGHWGIGENC) and 6) negative control target (BSA).

There was no apparent binding to MMP2, which appears due to MMP2 not binding to the plate. We will evaluate the impact of different detergents on binding of MMP2 to plates and repeat these experiments. Binding to MMP9 seemed to be equivalent in all experiments including the negative controls.

These experiments were repeated with MMP9, Grb2, and BSA (500ng each) as targets. Grb2 is a signal transduction molecule that binds to phosphorylated residues on the intracellular domain of ErbB family. Clone MMP9 was evaluated as the expected positive binding ligand and Grb2 clone as a negative control. As an additional control to the experiment, binding to purified Grb2 target by G1 (positive control) and the putative MMP9 binding clone (negative control) was performed. G1 is a peptide-phage clone that we previously developed that binds to the SH2 domain of Grb2.

Unfortunately, MMP9 clone, library, and clone IV092499-16 all bound similarly to MMP9. G1 bound to Grb2 as expected and showed good positive control to purified Grb2.

Further evaluation of clone binding is underway and may involve using APMA activated (aminophenyl mercuric acetate) MMP9.

Characterization of selected clones harvested from mouse tumor:

In vivo panning has the advantage of a multiplicity of targets for possible binding by the phage displayed RPL. This asset is also a problem downstream in terms of target identification and evaluation of ligand-target interactions. The method most readily available for determining the approximate location of ligand binding is to expose candidate peptide-phage clones directly on a histologic tissue slice prepared from the mouse tumor. We have learned that this technique is not straightforward and may not be an optimal technique to accomplish this goal. There are several variables that are difficult to control, for example, determining the optimal concentration of phage to place on the histologic specimen. As with conventional IHC with antibodies, each antibody must be evaluated for appropriate dilution. Even with antibodies known to bind to specific targets, an incorrect concentration will result in either false negative or false positive results. Determining appropriate peptide-phage clone concentration for IHC, particularly with unknown binding properties, is quite challenging.

In order to insure that we had low background from unbound phage in the blood, we confirmed that the method of mouse whole body perfusion methods were satisfactory using blue dye. We observed that superficial structures, extremities (tail, paws, nose) turned blue. The heart, kidney, tumor, and liver were all blue upon dissection. Blue staining of the lung and spleen were less conclusive. When the lungs deflated it was difficult to discern interior vs. exterior when cut in half. The chest cavity fills with dye from spillage of the perfusate (from the catheter inserted into the heart) and stains tissues in the chest cavity. Brain tissue was not blue, but vessels in base of skull (carotids) appeared blue. Repeat experimentation with more attention to chest cavity spillage demonstrated that vessels in the lung were in fact blue stained as were blood vessels in the brain.

Preliminary evaluation of binding of our MMP-associated clone to paraffin sections of the mouse tumor was performed during the last report period. The results were very inconclusive. Therefore we have generated a number of new positive nonMMP related controls so as to define a range of conditions that might be encountered with an unknown clone. A number of organ binding peptide sequences have been reported which were identified by in vivo peptide-phage panning in a mouse model. We have constructed fusion phage based on these reported organ seeking peptide sequences [5-7]. The following clones were prepared in the same way as the MMP fusion phage described above:

THP=tumor homing peptide=CNGRCVSGCAGRC
BHP=brain homing peptide=CGRECVRQCPERC
LHP=lung homing peptide=CLSSRLDAC

In the first experiment employing these clones, the tumor-homing peptide-phage clone (THP) was injected into an MRL tumor-bearing mouse. After 10 minutes, the heart was perfused with 20cc's Hanks Balanced Salt Solution (HBSS) until the liver blanched. The tumor, brain, lung, and kidney were harvested for IHC and titering. Tumor titers (TU's/mg tissue) were 6.5x and 1.6x higher than brain and lung respectively.Kidney titers however were 1.9x higher than tumor. No phage were seen by anti-M13 IHC (at same dilution that showed staining in toxicity studies).

In a subsequent experiment, brain-homing peptide-phage (BHP), lung-homing peptide-phage (LHP) and naïve library (Lib) were injected IV (1 x 10^8TU's) into each of three BalbC mice. As before, the phage was allowed to circulate 10 minutes, the heart perfused, and organs harvested. Brain, lung, and kidney were then subjected to IHC and titering analyses. Tissue titers for BHP showed 22-fold and 5.6-fold more phage in lung and kidney than in brain. LHP tissue titers showed lung to be 48x and 3.2x higher than brain and kidney respectively. However lung titers from the naïve library injected mouse showed greater amounts of infective phage (1.4x) compared to the LHP-injected mouse. IHC results on these tissues were negative for staining.

Regulatory issues and accomplishments:

Even though phage have been administered to humans by direct application to surface wounds, ingestion, and by intravenous administration, intravenous administration of a recombinant phage-displayed peptide library has not previously been reported. There has been extensive regulatory and scientific oversight of our research by a variety of important organizations. A substantial portion of the activities related to this grant during the past year has been completing tasks necessary to achieving approval by the various regulatory committees and organizations.

The most important first step in this process was the approval by the Food and Drug Administration of an Investigational New Drug for administering the phage-displayed RPL to human cancer patients. Follow up documentation and modifications of the original IND application were submitted July 6, 2001. See appendix for additions and corrections.

Approval by the University of Vermont Committees on Human Research in the Medical Sciences (CHRMS) was also an extensive process and represented a considerable portion of the activities related to this grant. Due to the unique nature of this protocol, the CHRMS required an extensive review. Following full review of our responses to the CHRMS questions and proposed changes, the protocol has been approved on February 7, 2001. In addition, as required by the CHRMS an ombudsman has been arranged that will meet with all patients entering this protocol. The assigned ombudsman is the immediate past chair of the CHRMS. The CHRM is also requiring a 30day period of time to elapse between each patient accrued to this protocol. At the end of 30 days a written summary of that individual case must be submitted, evaluated, and approved by the committee prior to enrolling the next patient. A copy of the approved protocol is attached as Appendix.

The National Cancer Institute requires that all Comprehensive Cancer Centers convene a Protocol Review Committee (PRC) in order to evaluate the scientific merit of all clinical protocols. The Protocol Review Committee of the University of Vermont extensively reviewed our protocol and a scientific exchange ensued. Preparation of the protocol for submission to the PRC and addressing the questions were all performed during the second year of this grant. The PRC has given provisional approval to proceed with the proposed human study on February 13, 2001.

The General Clinical Research Center (Grant RR00109) of the University of Vermont is supporting this protocol both materially and scientifically. The GCRC provides support for selected clinical trials. For our clinical trial the GCRC will be providing administrative support, nursing support, clinical supplies, and clinical space. Accomplishments during this grant report period include establishing the precise workflow in the GCRC to accomplish this protocol. The GCRC protocol review committee first evaluated the protocol. The committee then addressed a

number of comments of the GCRC committee leading to full approval of the protocol. A detailed workflow plan has been established and tasks assigned. Complete mock runs have been performed in order to test the protocol flow and to insure that all the appropriate resources are allocated. The workflow diagram is attached in the appendix.

In the GCRC, patients will undergo pre-screening tests to confirm eligibility. Blood (volume 20 ml) will be drawn to measure hematologic, renal, and hepatic function. A blood test will be required to determine that a woman of childbearing age is not pregnant prior to participation in this study. Women with a documented history or status (hysterectomy) that excludes the possibility of becoming pregnant will not require the pregnancy test. History and physical exam will include methods for determining whether a patient has pulmonary impairment or signs or symptoms of brain metastases. Pulmonary function studies and/or brain MRI or CT scan will be performed to confirm or rule out any suspicious clinical findings. Eligible patients will be given a premedication plan (prescriptions and instructions) prior to the first procedure to decrease the risk of an allergic reaction.

Patients will be admitted to the GCRC. An intravenous line will be placed. Baseline vital signs (blood pressure, pulse, temperature, and respiratory rate) will be determined before infusion of phage and every 15 minutes during infusion and for 2 hours after infusion. The patient will be under constant supervision and equipment, medications, and personnel capable of treating allergic (including anaphylactic reactions) will be immediately available.

Ideally, we will screen our RPL(s) three times in the same patient. In the unlikely event of toxicity with naïve libraries, no further pans will be performed. In the event that only amplified phage show evidence of toxicity, no further pans will be performed.

A phage displayed RPL pool will be prepared and tested according to FDA standards. Before phage injection, one sample of tumor tissue from the patient will be biopsied, snap frozen and stored for later testing of selected clones for tumor binding affinity. A small portion of the tissue sample will be fixed in formalin and submitted for histological analysis and confirmation that harvested tissue is tumor tissue.

The library will be diluted in 100 - 250 ml saline and infused intravenously over approximately 10 minutes into a breast cancer patient. Collection of tumor-bound phage will be performed by obtaining small amounts of tumor tissue. Tumor-tissue acquisition (biopsy) will occur in the procedure room of the GCRC approximately 10 – 60 minutes post infusion. Standard surgical technique for performing a surgical biopsy will be utilized for the biopsy procedures. It is intended that the biopsies be as small as possible and will be on the order of 1.0 gram of tissue. The method of biopsy will be either incisional, excisional, or core depending on the location of the tumor.

The tumor will be rinsed to remove blood, the tissue will be ground, and *E. coli* will be added to amplify phage. A small portion of the tumor will be fixed in formalin and submitted for histological analysis to confirm that harvested tissue is tumor tissue. Phage will be eluted from tumor cells and amplified. Phage will be amplified and quantified by titering, with results available within 12 - 24 hours. Enriched phage will be prepared as was described above for initial infusion and will be reinjected as soon as possible (1 to 2 days). Phage administration, tumor harvest, and amplification will be repeated a maximum of 2 times for a maximum of three screenings in one patient. Screening will completed within 10 days and ideally in less than 7 days to avoid patient Ab response to phage.

The primary end points for dose escalation will be toxicity (stopping) and phage isolation from tumor (continuing). The definition of successful phage isolation will be 1) the presence of

phage in the tumor after each tumor tissue harvest, and 2) the presence of at least one three-amino acid consensus sequence in clones from the final pan.

The first patient will receive 10^{9-10} phage, and phage isolation from tumor will be evaluated. If no phages are isolated from the tumor, then a second patient will be treated with 10^{11} phages and attempt isolation from tumor. If the dose of phage must be escalated to 10^{12} or greater, three patients will be treated at each dose level to assure safety of the intravenous injection of that concentration of phage. Increase in the phage concentration will not be $> 10^{1}$ at any step in the dose escalation as based on titering. Once an acceptable amount of phage is observed in the tumor specimen, amplification and readministration of phage to patients will be initiated with each patient to receive up to three injections over a period of approximately seven days. The concentration of phage used for reinfusion will not exceed the initial concentration used for that patient unless safety of the higher dose of phage infusion has been demonstrated previously in three patients.

Phage will be amplified from the final tumor harvest. At least 20 clones will be selected and sequenced. If all patients at that phage dose level have consensus sequences (indicating sufficient copy number of phage at time of initial phage administration) no further dose escalation will be performed.

Throughout the screening process patients will be carefully evaluated for adverse reactions. Injection of phage will be stopped immediately in any patient if Grade II or greater allergic reactions (NCI CTC Version 2.0) occur. Further doses of phage will not be administered to that patient. Other NCI-CTC toxicities grade III or greater at any dose level are grounds for discontinuation of phage dose and for escalation of phage dose. Patients will be evaluated approximately one month after completion of phage injections(s) for delayed toxicities.

Peptides displayed by phage isolated from tumor tissue will be routinely analyzed for both consensus amino acid sequences and tumor-binding. Clones that bind to tumor tissue will be assessed for binding to non-tumor tissue. A panel of different non-tumor tissues will be obtained from the Vermont Cancer Center Tissue Procurement Facility. Immunohisotochemistry with anti-transferring receptor mAb will be used as a positive control to assure tissue and assay reliability.

Any consensus sequences identified from phage eluted specifically from the tumor tissue will be excellent candidates for tumor-specific peptides. Promising peptides will be synthesized and tested for tumor specificity. Peptide binders we identify by whole body screens, almost by definition, are likely to be stable in serum and generally stable *in vivo*, a major advantage to this technique.

The protocol has also been prepared for and submitted to the Surgeon General's Human Subjects Research Review Board (HSRRB). Extensive communications occurred regarding preparation of this protocol and a number of protocol modifications made at the request of the -(HSRRB) committee. The final approval is pending.

In summary, this is a complex human patient protocol that has required extensive review by several regulatory committees. Each committee has required a unique set of proposal documents. Each committee has required written response to a variety of issues. All of these issues have been addressed and have either been approved by the respective committee or are in the final stages of response.

KEY RESEARCH ACCOMPLISHMENTS:

- Construction of mouse brain-homing, lung-homing, and tumor-homing phage clones based on reported peptide sequences. In vivo assays in mice were performed with the expectation that these clones would provide positive controls. These clones did not home to mouse brain tissue at levels greater than negative control phage.
  - Tumor-homing peptide (CNGRCVSGCAGRC)[5]
  - Lung-homing peptide (CGFECVRQCPERC)[6].
  - Brain-homing peptide (CLSSRLDAC)[7]
- Demonstration of immune response in mice to administration of phage-displayed RPL.
- Initial evaluation of putative MMP peptide ligands complete. No evidence of MMP binding was obtained with existing methods.
- Approval by the Food and Drug Administration to perform human studies of administration of phage-displayed RPL and tumor panning (Investivational New Drug approval (BB-IND#9145)
- Approval by the University of Vermont Committee on Human Research in the Medical Sciences for the human cancer patient study of administration of phage-displayed RPL and tumor panning.
- Approval by the University of Vermont Comprehensive Cancer Center Protocol Review Committee for human cancer patient protocol to study administration of phage-displayed RPL and tumor panning.
- Approval by University of Vermont General Clinical Research Center Advisory Committee for human cancer patient protocol to study administration of phage-displayed RPL and tumor panning.
- Approval for support by University of Vermont General Clinical Research Center. Extensive protocol development and task assignments have been completed. Mock patient entry "dry run" performed to insure that all resources present and appropriately assigned.
- Response to Office Action of Patent on In Vivo method of phage display submitted to the United States Patent Office.
- Initial development of human IgG immune response assay. In preparation for our clinical trial, an ELISA was developed to evaluate human IgG levels. Over the course of several assays, we determined the correct dilution for the antibodies, goat anti-human IgG HRP and donkey anti-rabbit IgG HRP. Using the correct antibody dilutions, another ELISA was performed using human serum from a normal volunteer (without exposure to a phage library) as a negative control. We determined that human serum diluted 1:1000 gave a response equivalent to PBS. Additionally, we demonstrated that the phage library applied to the ELISA plate as a target was detectable by Rabbit anti-M13 IgG after incubation with donkey anti-rabbit IgG HRP, a control we routinely employ to verify our phage have bound to the microplate.

REPORTABLE OUTCOMES:

- Investigational New Drug approval by the Food and Drug Administration.
- As reported in the last Annual Report, the activities of this grant were submitted as part of the University of Vermont Comprehensive Cancer Center Grant Application. Our section was scored high and the Cancer Center Grant has subsequently been approved for five years of funding.

- A manuscript detailing the mouse toxicity data and the initial results of peptide ligands obtained from the harvested tumors has been submitted.

CONCLUSIONS:

All necessary amendments and responses have been made to the Food and Drug Administration resulting in approval of an Investigation New Drug for human cancer patient studies of systemic administration of phage-displayed RPLs. This is a very important milestone that had to precede all other regulatory committee approvals. Since approval by the FDA, approvals to proceed with the human clinical study have been obtained from the University of Vermont Comprehensive Cancer Center Protocol Review Committee, the University of Vermont of Human Subject Committee, and the University of Vermont General Clinical Research Center. Extensive planning and resource allocation has been established with the UVM GCRC and detailed plans including mock patient entry have been accomplished. We are prepared in this third year of grant support to safely enter patients into this clinical study.

We will continue to develop methods related to evaluation of clones obtained from in vivo panning. Based on our experience and others it is expected that initial clones may have low affinity to the target. When evaluating clones from in vitro panning experiments there is generally an ample supply of the target molecule in purified form. Promising clones are evaluated by ELISA. This method of analysis is not currently possible with clones obtained by in vivo panning since the target molecule is unknown. The method that we have been using to evaluate candidate clones from the mouse experiments is based on histochemistry. A histologic slide is prepared of target tissue and candidate clones allowed to incubate on the slide. After rinsing, antiphage antibodies are applied in order to determine whether a candidate clone binds to the target. This same technique, assuming that positive binding is demonstrated, should allow evaluation of the specificity phage clone binding. This procedure is qualitative at best and is highly dependent on a number of variables such as amount of rinsing, affinity of clone, amount of target molecule present on histologic slide, phage clone concentration, and method of tissue fixation. We are continuing to try and refine this histologic method of evaluation and considering a variety of alternative methodologies.

Evaluation and characterization of promising clones that have consensus sequences harvested from panned tumor specimens remains an important obstacle. Since it is expected that further modification of candidate peptides will be necessary in order to improve ligand affinity, development of methods to evaluate and characterize peptides is an important goal.

We are highly enthusiastic about the overall research approach. This is based on the observation that 1) there was minimal to no toxicity of phage-displayed RPLs despite repeated administration and 2) several peptide consensus sequences were obtained following serial panning of a lymphoid tumor. As reported in the previous Annual Report, some of the consensus sequences were nearly identical to reported peptide sequences obtained by panning over purified MMPs. This suggests that important targets can be identified in a very rapid period of time by in vivo panning and harvesting of phage clones from excised tumor specimens. We remain highly optimistic that peptides with significant affinity to tumors in breast cancer patients can be obtained. These peptides will serve as ligands to improve the functional delivery of cytotoxic agents in patients with breast cancer. By this method we hope to increase the tumor cell kill and decrease the toxic side effects of systemic therapy.

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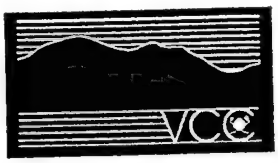
APPENDICES



In Vivo Selection of Ligands for Targeted Therapy: Breast Cancer

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1.0 INTRODUCTION

A major problem in the treatment of breast cancer is that present therapies lack specificity for tumor cells and are extremely toxic to normal cells. The development of therapies with high specificity for tumor cells is an utmost priority in breast cancer research. Exciting progress has been made in the elucidation of key molecules found specifically overexpressed or underexpressed in breast cancer cells. However, effective ways to exploit these tumor-associated targets for therapy have not yet been developed. A means to specifically direct therapeutic agents to these defined molecular differences is critically needed. Many high molecular weight targeting agents, antibodies in particular, have been identified which are specifically directed against tumor-associated molecules. However, coupled to cytotoxic agents or alone, the performance of antibodies (Abs) or Ab fragments in clinical trials has been disappointing [22, 45, 46, 48, 49]. Results with Herceptin, an antibody against the clinically important breast cancer target ErbB2, have been more promising than most antibody trials, and confirm the value of ErbB2 as a target. However, the clinical responses with Herceptin are far from ideal [15, 75]. Failure of antibodies in the clinic is likely due to the unfavorable pharmacokinetics, lack of tumor penetration, and immunogenicity of molecules this large, as well as their non-specific uptake by the reticuloendothelial system [16, 33, 41, 57]. Smaller single chain Fv (sFv) Ab fragments have been developed with high affinity to ErbB2 [70] and it will be interesting to see how these molecules perform in the clinic. However, the vast majority of effective drugs are of much lower molecular weight than sFvs (25kD) and the discovery of smaller tumor-specific ligands would be extremely valuable. As has become apparent with the Herceptin trials, and also in Judah Folkman's promising work with antiangiogenesis factors, large proteins are also difficult to synthesize in amounts necessary for clinical use. Furthermore, many of the most promising cancer-specific targets are intracellular or intranuclear. Antibodies are not likely to be effective against these intracellular targets. Because of this disappointing progress in tumor-targeted therapy over the past decades, it is clear that dramatically innovative approaches are needed.

Our lab is attempting to identify novel *small* ligands (1-2 kD) that bind specifically to tumor cells. Small ligands may have therapeutic activity alone, as does Herceptin, presumably by inhibiting a target molecule that actively plays a role in carcinogenesis. Small ligands can also be coupled to cytotoxic agents and used to mediate the specific destruction of tumor cells, even if their target molecules do not play an active role in cancer progression, as long as they are specifically present on the tumor (or on blood vessels specifically supplying tumor.) Ligands much smaller than antibody fragments may have important advantages in targeted therapy including improved tumor to non-tumor uptake ratios, better penetration of solid tumors, and non-immunogenicity. Small molecules are also easier to synthesize in the large amounts necessary for clinical use, are less likely to interfere with the effects of conjugated cytotoxic drugs, and may have improved specificity as there is less surface to interact non-specifically with other body components.

Large libraries of small compounds are a rich source of small ligands that may target tumors. Several types of these libraries, which consist of millions or even billions of different peptides, oligonucleotides, or synthetic molecules, have been constructed and used to isolate small ligands or lead compounds to many targets. The construction of libraries like these and their use in the identification of specific ligands, known as combinatorial technology, has revolutionized the field of drug discovery [28]. This proposal describes the use of this technology to identify small *peptide* ligands that will specifically bind to tumor cells and not to normal cells.

Although peptides have traditionally been discounted as potential therapeutics due to an assumption of their instability *in vivo*, peptides can form an almost infinite number of shapes and are exactly what nature uses to specifically target molecules both intracellularly and extracellularly. Many peptides have important biologic functions and potent *in vivo* activities. Furthermore, the exciting work of Ruoslahti et al [2, 60, 61, 65] has demonstrated that many peptides are stabile enough in serum to home specifically to tumors and to various organs. Elegant

experiments from Affymax [18, 90] also demonstrated that, with minimal modification, peptides are capable of strong binding and effective agonist activity *in vivo* to cell surface receptors. Furthermore, small peptide ligands which are identified to tumor targets by *in vitro* screening, even if lacking *in vivo* stability, can be modified and/or used as a prototype in order to develop a small molecule which will be more stable and effective *in vivo*. We have already identified such a small peptide ligand to a potential tumor target, Grb2, and our collaborators have modified it chemically such that it retains binding activity in cell lysates [59]. Cyclic peptides are more stable *in vivo* and are often more selective for their targets. Many peptide libraries, including those used in our lab, are biased for cyclic peptides. D-amino acids, non-natural amino acids, and pseudo-peptide bonds may also confer greater *in vivo* stability. A novel and intriguing method of obtaining mimetics that may be more stable *in vivo* involves using a D-amino acid synthesized target. Screening with the D-amino acid target can result in the identification of D-amino acid peptide ligands to natural L-amino acid targets [71]. Peptide structures can also be readily determined by NMR and used to model peptidomimetics that may be more stable *in vivo*. The structure of peptide ligands can also provide important information about the structure of both their receptor targets and the natural ligands of those targets. Identification of peptide ligands can also facilitate the discovery of natural ligands to orphan receptors such as ErbB2. Other advantages of peptide ligands for tumor targeting are that they can be easily synthesized in the large amounts needed for clinical use, their chemistry is well known, and conjugation methods are routine.

Peptides and peptidomimetics are very promising targeting agents because they can potentially bind targets with the same exquisite specificity as antibodies, and are likely to have far more favorable pharmacokinetics. Peptides can have direct agonist or inhibitory activity on therapeutic targets. In another project, our lab is attempting to identify peptides that directly inhibit the dimerization of ErbB2, an effect that may result in therapeutic activity. Alternatively, peptide motifs can be used to direct other agents that have therapeutic activity, such as cytotoxic drugs, immune modulating agents, ribozymes, and gene therapy delivery systems such as liposomes or viral particles, to a specific molecular target. In a remarkable recent report, a short peptide sequence was grafted onto a 41kD protein capable of inhibiting growth factor receptor signaling [69]. The peptide allowed the protein to penetrate the cell membrane and to be delivered into the cytosol from the extracellular environment without detectable proteolysis. The delivered protein was successful in inhibiting growth factor signal transduction. Peptides can also potentially target imaging agents for diagnostic purposes.

Using a combinatorial approach, large random peptide libraries (RPLs) have been constructed in several systems. The RPLs described in this proposal have been or will be constructed in a phage-display system [19, 21, 83]. Phage-displayed and other biological RPLs are particularly powerful in that the peptides are physically linked to their encoding DNA. Because DNA is easily amplified for sequencing, one binding peptide out of millions can be determined. Phage-displayed libraries are made using filamentous phage that infect and multiply in *E. coli*. Each phage particle has five copies of a minor coat protein (pIII) located at one end. Random synthetic DNA is inserted into the gene coding for pIII so that the foreign DNA is expressed at the free N-terminus of pIII as random peptides. In this system, up to 5 copies of each peptide are physically "displayed" by each phage particle. (Another commonly used phage-display system employs the major coat protein and displays several hundred peptides per particle.) Each phage particle displays a different peptide. A phage particle bearing a peptide which bonds to a target can be isolated using affinity selection and is easily amplified in *E. coli*. After amplification the phage DNA can be sequenced to deduce the identity of the displayed peptide. The small size of the library particles allows manipulation of millions of different potential binding units in a few microliters.

Phage-displayed RPLs have been used by our lab and others to isolate small ligands, some with nanomolar and even picomolar affinity, to a large variety of targets including several potential tumor targets and other clinically important targets [2, 18, 60, 61, 65, 90]. One example of the use of small peptides (8 and 12 mer) in targeting tumors has been reported by Renschler et al. [66, 67]

who used phage displayed RPLs to identify peptides that bind to the antigen binding receptor of B-lymphoma cells and induce apoptosis *in vitro*. Most of these ligands have been identified using *in vitro* screening techniques: binding purified target protein to a matrix, incubation of the immobilized protein with the peptide-phage library, washing away non-specific binders, elution of specifically bound phage, followed by phage amplification and DNA sequencing to determine the identity of the peptide responsible for binding activity.

This proposal describes the use of phage-displayed RPL technology to identify small peptide ligands to breast cancer-specific targets by *in vivo* screening in breast cancer patients. In future studies, these peptides will be modified if necessary to optimize *in vivo* stability, coupled to cytotoxic or other therapeutic agents, and used to mediate the specific destruction of breast tumor cells. Proof of concept for our project is found in several exciting recent reports [2, 18, 60, 61, 65, 90], one which describes *in vivo* screening of RPLs similar to ours in mice bearing human tumor xenografts and the identification of peptides which home specifically to tumor blood vessels. Administration of peptide-doxorubicin conjugates to tumor-bearing mice resulted in a marked decrease in doxorubicin toxicity, selective tumor destruction, and excellent animal survival [2, 4]. The same group has also identified peptides that bind preferentially *in vivo* to at least 10 different organs [61, 65], further demonstrating the powerful ability of small peptides to home to specific molecular "addresses" in the body. As mentioned above, Affymax has also identified peptides from libraries similar to ours that bind with high affinity *in vivo* to clinically important cell targets. These important findings have introduced a whole new field of exploration in the search for more specific and effective cancer therapeutics.

We are in a unique position to perform the novel *in vivo* RPL human screening experiments described in this proposal. We have extensive experience with phage RPLs and have successfully developed binders to several potentially clinically useful targets. The PI has extensive clinical experience in the IV injection of experimental compounds such as radiolabeled monoclonal antibodies and the intratumor injection of technetium colloid materials. In vivo RPL screening experiments [2,4,61,65] are certainly among the most intriguing and novel in cancer research today. We feel it is important and urgent to pioneer similar techniques in humans, as the identification of ligands that recognize mouse endothelial cells may not be relevant to treatment of human breast cancer.

In vivo RPL screens in humans offer several potentially critical advantages over *in vitro* screening including:

1) Tumor targets will be in their native conformation with all their human post-translational modifications. Screening targets in their native *in vivo* conformation may be especially important in light of the following: host endothelial targets may be among the most promising "tumor" targets available [10-12, 27, 37, 60] [4] and "the phenotype of endothelial cells is unstable and likely to change when the cells are removed from their microenvironment [3, 8, 65]." Therefore, IV injection of RPLs *in vivo* may not only be the *optimal* method of presenting these important endothelial targets to the library- it may be the *only* effective method. Host targets, which are genetically stable and homogenously present on target tissues, are particularly attractive due to the heterogenous nature of most cells of a tumor, which may be difficult to target with even a combination of drugs. However, targets located directly on tumor cells are also most advantageously presented in their native conformation.
2) Inherent selection of peptides that recognize *specific* targets due to efficient "subtraction" of library clones which bind to normal tissue during exposure of the injected library to the entire body. Efficient removal of library clones that bind normal tissue is essential to the recovery of tumor-specific clones. It would be difficult to "subtract" all clones that bind to every possible normal tissue by *in vitro* subtraction methods, as this would require having fresh tissue samples from dozens of human tissues harvested from a cadaver. Subtraction with fixed tissues may not give satisfactory results since all targets do not retain native conformation during tissue fixation. Obviously, *in vitro* subtraction with all possible

normal tissues, fresh or fixed, would be impossible to do with the normal tissue of the same patient being screened. Rajotte *et al.* [65] noted how their *in vivo* screening method "surprisingly...consistently yielded tissue-specific homing peptides" and attribute this success to efficient whole body "elimination of nonspecific phage." Further evidence that *in vivo* screening methods may be more effective than *in vitro* methods in the selection of specific ligands to cell surface targets is supplied in an interesting report by Barry et al [5]. Using whole cells to pan *in vitro*, peptides were identified which bound to many cell types rather than peptides that bound specifically to the cell type used for screening.

3) Inherent selection of *only* peptides that are stable *in vivo*.
4) Inherent targeting of *only* targets that are stable *in vivo* and which are capable of stablely binding ligands *in vivo*.
5) Purification or prior knowledge of particular targets is not necessary.
6) Potential elucidation of novel tumor targets. Targets are not influenced by immunogenicity as with targets defined only by monoclonal antibody development [5].

Screening whole cells or cell extracts in vitro can also achieve the latter two advantages; however, many of the important advantages will be lost using this technique. Similarly, while direct injection of tumor with RPLs for screening is certainly possible, the important advantage of whole body elimination of normal tissue binding clones will be lost, and the chance of exposing the library to the important apical surface of endothelial cells will be greatly decreased. Systemic injection may well be the most effective way to identify ligands with a good tumor/control tissue-binding ratio. For example, in previous in vivo screening experiments, a peptide with a RGD motif binds integrins three orders of magnitude better than an NGR peptide. However, the NGR peptide had a tumor/control tissue homing ratio three times better than that of the RGD peptide [2].

The following proposal will extend *in vivo* screening techniques in the following ways to optimize its success in identifying small tumor-specific ligands in humans:

1) Identification of *human* targets. Ligands to mouse endothelial targets are not likely to be as effective or effective at all for human targets. In the previous *in vivo* tumor screening study in mice, many of the tumor-homing peptides contained an RGD amino acid motif, as did one of the two peptides assessed for their ability to direct doxorubicin to tumors [2]. Although integrins and related receptors are very promising tumor targets, and many integrin binding sequences contain an RGD motif, the regions flanking RGD are critical to the specificity of integrin binding. Optimal flanking regions of these integrin binding peptides are not likely to be identical for the recognition of both mouse and human integrins on newly developed endothelial cells, as mouse and human integrins are not identical as assessed by GCG analysis. Peptides identified by human *in vivo* screenings are far more likely to yield specific and high affinity binders to human tumors.
2) Identification of targets in a realistic setting. The mouse model was a xenograft model, and did not involve mouse tumor. This artificial model may yield results not applicable to the eradication of natural tumors.
3) By using a large panel of libraries that offer a variety of structural contexts for peptide presentation, we believe we may isolate peptides that bind to other promising breast cancer targets as well as to tumor-associated integrins. It is likely that a successful therapeutic regimen for advanced cancer will require a "cocktail" of anti-cancer compounds. Targeting a single cancer target is not likely to effect a long-term favorable therapeutic response. In addition, there is evidence that other sequences may bind as tightly and more specifically than RGD sequences to integrins [9]. It is also possible that RGD and NGR peptides [2] may bind to too many targets to be specific enough. Even low affinity binding can have significant biological consequences, particularly with avidity effects, as is the case with integrin binding to fibronectin. Furthermore, a large panel of libraries which present a vast number of peptides presented in a variety of structural contexts is much more

likely to yield a high affinity binder to any given target [7]. We will also employ several innovative RPL technical improvements, developed by both our lab and Affymax.

4) We will attempt to identify peptide ligands to endothelial targets by harvesting tissue approximately 10 – 60 minutes after library injection.
5) We will attempt to assay peptide binding directly rather than via competition with peptide-phage as done in previous *in vivo* screening experiments. This will allow us to more accurately determine the degree of specificity by immunohistochemical analysis of peptides on tumor tissues and a large panel of normal tissues.
6) By performing multiple screens in one person, rather than serially as was done with mice, we may determine which targets are *unique* to a particular individual. (Important *generic* targets will be identified by similarities in consensus sequences identified from different patients in whom complete multiple screenings were performed, or by serial screens between different patients.) Even though screens will be initially performed on patients with advanced disease, it is very conceivable to establish a profile of ligands against the majority of newly diagnosed patients with breast cancer immediately prior to definitive surgery. This would allow design of systemic adjuvant therapy most appropriate to each patient.
7) Very importantly, we will carefully investigate toxicity during phage injections and screenings. Toxicity in human cancer patients during *in vivo* RPL screening has not previously been studied.
8) We will test several screening designs, including multiple screenings in one patient to determine the safest and most effective screening protocol.

A large portion of the following proposal is designed to examine possible toxicity during *in vivo* screening. We do not expect the screening procedure to cause toxicity as bacteriophage have been injected intravenously in humans and even neonates for over 30 years in approximately 3000 patients with essentially no side effects. This is extensively reported in the literature [13, 36, 58, 62, 85] Bacteriophage are injected into humans IV routinely for analysis of antibody responses.

There has also been extensive use of over 250 strains of bacteriophage, including 39 that infect *Escherichia* bacteria, which were administered orally or locally for treatment of infection [76-82]. Not only were "side effects" described as "extremely rare" (3 allergic responses out of 138, with no prior endotoxin testing), the phage treatments were often effective in eliminating the bacterial infection.

Bacteriophage are known to specifically infect only bacteria, and each bacteriophage strain infects only a very narrow range of bacterial species. In addition, it has long been known that ssDNA is expressed poorly or not at all in mammalian cells. The presence of phage DNA in human cells was examined in the antibody analysis studies mentioned above several months after injection and none was detected. Therefore the possibility of bacteriophage "infecting" eukaryotic cells in any way is highly unlikely. We do not have a PCR technique developed and available at this time to monitor this possibility. However, 10mls of blood will be drawn from each patient prior to initial infusion and 6 – 8 weeks following the last phage injection. The serum will be stored at –20 degrees C for future PCR testing should this be judged necessary.

Dr. Hans Ochs, an immunologist who is one of the pioneers of using phage ϕX174 for antibody response analysis, is an enthusiastic collaborator on this project. Dr. Och's group has had an IND from the FDA for over 20 years for this procedure. We will have his expertise available to us for consultations on human phage injection and he will provide us with his strain of phage for library construction if necessary. Not only has the work of Och's et al been extensively documented for non-toxicity, their experiments give us an accurate estimate of the time we have available to screen before we expect immune system interference. Our library is constructed in a strain of bacteriophage different than the strain used for antibody response analysis, although they both infect *E. coli* exclusively. However, filamentous phage injection is not likely to cause toxicity, as there have been numerous reports of the injection of filamentous phage into mice for

several other purposes including hybridoma development. There are several groups investigating the use of filamentous phage particles as potential vaccine delivery agents, with numerous preliminary studies in mice. At least one of these studies reports IV injection of a very large number ($2x10^{12}$) of phage particles with no toxicity reported, although toxicity was not explicitly addressed [32]. The mice survived at least 3 to 4 days when they were sacrificed in order to harvest spleen cells for hybridoma development. This proposal has been carefully designed to detect any possible toxicity.

In addition to the available literature which supports the safety of performing human studies, we have conducted preclinical toxicity testing in mice to prepare for this human research proposal. The methods of testing were done in consultation with the FDA and were specifically designed to mimic the proposed human studies. Based on the demonstrated lack of toxicity in preclinical testing, the FDA has granted the University of Vermont an Investigational New Drug approval to proceed with human studies. The preclinical studies are summarized below and the complete FDA application is included as an appendix(BB-IND 9145).

Four in vivo studies (Study I-IV) were designed and implemented to assess the toxicity of phage random peptide library (RPL) screening in a mouse model. The toxicity studies were designed to mimic as closely as possible the scheme that will be used in phase I clinical trials. A total of 31 mice were injected with 3 different preparations of peptide phage (naïve peptide-phage, peptide-phage amplified once from tumor (φ Amp1x), or phage amplified twice from tumor (φ Amp2x)), and were monitored daily for three days or three weeks after phage injection for signs of toxicity. The FDA had suggested that 3 day and 3 week time-points for organ harvest would allow us to evaluate both acute and chronic toxicity of peptide-phage injections (See individual study sections for details). At the end point of each study, 10 organs were harvested from each mouse and subjected to three analyses: hematoxylin & eosin staining (H&E) to assess pathology; immunohistochemistry (IHC) to look for the presence of phage particles (or at least intact phage coat proteins), which are not necessarily infective; and phage titering to determine the number of infective phage remaining. A brief description of each study follows. Complete details for each study may be found within each study section of the IND.

- Study I was designed to assess toxicity in mice, either 3 days or 3 weeks, following a single IV injection of naïve peptide-phage. The number of each peptide present at this stage (approximately 20 picograms) is so small that any toxicity which might result would most certainly be caused by the phage particles alone. (Refer to Diagram of Study Design: Study I in Study I section of the IND.)

- Study II was designed to assess toxicity in mice following a single injection of phage which had been collected from a tumor and subsequently amplified. This study evaluated whether any toxicity resulted from peptide-phage particles enriched for tumor binding (Refer to Diagram of Study Design: Study II in Study II section of the IND).

- Study III was designed to study toxicity in mice following three sequential IV injections of three different peptide-phage preparations: naïve peptide-phage library, peptide-phage amplified from a tumor in another mouse (φ Amp1x) and φ Amp1x passaged again through a second tumor (φ Amp2x). (Refer to Diagram of Study Design: Study III in Study III section of the IND)

- Study IV was designed to study toxicity in tumor bearing mice following three sequential IV injections of three different phage preparations in the same mouse: (1)naïve library, (2)phage amplified from a tumor excised from the same animal (φ Amp1x) after injection of naïve peptide-phage library, and (3)phage amplified from a second tumor excised from the same animal (φ Amp2x) after injection of φ Amp1x. Each injection occurred on separate days, in the order listed, followed 10 minutes later by excision of tumor. (Refer to Diagram of Study

Design: Study IV in Study IV section.) The purpose of Study IV was to carry out in vivo screening in animals using a protocol nearly identical to the planned clinical protocol. Study IV examined the toxicity of the complete in vivo screening clinical protocol and also allowed evaluation of whether the procedure can enrich for certain phage-displayed peptides, some of which may bind specifically to tumor tissue.

Three strains of mice (acquired from Jackson Labs in Bar Harbor, Maine) were used for the four toxicity studies described above: FVB, BalbC, and MRL/MpJ-fas$_{LPR}$(MRL). The FVB and BalbC mice are normal strains that have intact immune systems. However, the MRL mice develop massive lymph node enlargement, or lymphoproliferative disease, beginning around 8 weeks of age. Because their lymph nodes become markedly enlarged (tumors) these mice were chosen to provide us with tumors targets for our in vivo screening. While these mice have the advantage of producing tumors for screening, they have the disadvantage of dying rather early (and somewhat unpredictably, 3-5 months in our experience) compared to other strains of mice. Therefore, it was difficult to predict which mice were most likely to remain alive for the entire duration of the study, as many died even with no treatment. Study mice needed to have at least three tumors for our studies, yet, ideally, were healthy enough to survive 3 surgeries within 5 days, and live to the 3-week endpoint (as in Study IV).

Overall findings: (Please refer to individual Study sections in the IND for complete detail.)

- **Survival**: Of the 31 phage-injected mice in our studies, all but one survived to the endpoint. The mouse that died before the study endpoint was one of the three surgical mice (#3), which died while under general anesthesia following the removal of tumor during the second surgery. (Refer to the Appendix for Survival Surgery Protocol.)
  Note that there were 32 planned phage-injected mice. However, one of these (see Study II) died in a restraint while preparing the animal for injection.

- **Gross appearance**: All non-surgical mice (n=28) appeared normal for the study duration as observed by activity level, appearance of coat, and posture. The mice that had surgeries to remove tumors (n=3) were somewhat less active the first day or two following each surgery. As discussed above, surgical mouse #3 died during the second surgery, therefore there are limited observations for this animal. (Refer to Study IV for more information.) Additionally, the surgical mice chewed and pulled their sutures; Mouse #2 removed its sutures to the point of opening an incision. The incision was cleaned and antibiotic ointment applied daily until the wound healed. Surgical mice (#1 and #2), which were subjected to a complete phage screening, similar to the clinical protocol except with more extensive surgery and anesthesia, progressed well to the end of the study.

- **Weights**: Mice were weighed daily, with the exception of some weekend measurements that are noted in the study sections. While we have not performed a statistical analysis of the data, generally the mice appear to have either maintained or gained weight (apparently, compared to controls, consistent with normal growth) throughout the course of each study with the following exceptions. One of the two groups of mice in Study II (mice injected with j Amp1x) dropped an average of 9.1% (n=7) of their body weight on the day following injection with phage, but recovered from this weight drop by the following day. The surgical mice (Study IV) dropped about 8% of their body weight in the days immediately following surgeries and stabilized. This is not surprising in light of the fact that they underwent general anesthesia 2-3 times in a short period of time, and had large tumors removed that accounted for up to 3% of the their body weight over the course of 3 surgeries. For example, surgical mouse #2, in Study IV, weighed 47.6 g on the first day of surgery, and had 3 tumors removed in 5 days weighing 600 mg, 403.6 mg, and 431.8 mg respectively.

- **Phage titers**: Phage titers were performed to determine the number of infective phage present in tissues. On the day of harvest, tissues samples from each of 9 organs and in some cases blood, were collected. The tissues were weighed prior to being homogenized and incubated with *E. coli* for titering. (The titering procedure is described in detail in the Appendix.)

At three days most blood and tissues were highly positive for infective phage . At three weeks following phage injection, regardless of preparation (naïve, φ Amp1x, or φ Amp2x) all tissues were negative for infective phage in all mice except one: surgical mouse #2. Surgical mouse #2 had some phage detected in most tissues at three weeks, although much less than the number typically detected in tissues three days after injection. The only tissue that did not have infective phage detected at three weeks in this mouse, interestingly, was tumor tissue.

- **IHC**: Slides for IHC were prepared by the Histology Department at FAHC according to their protocol (see Appendix) using rabbit anti-M13 primary antibody (Sigma B7786, lot 038H4885) at a concentration of 0.73 μg/ml, a 1:10,000 dilution of the stock provided by our laboratory. Most tissues were negative for phage particles at three weeks.

- **H&E:** All tissues in all studies were found to be of normal histology for the given strain except for 3 out of 4 FVB livers in Study II. The project pathologist found some histological differences inherent to the strain of mouse after looking at control animals. Specifically, MRL mice typically have: (1)enlarged lymph nodes, (2)enlarged spleens with markedly expanded white pulp with lymphoproliferative disorder, and (3)glomerulonephritis in the kidney. BalbCs also had a strain-specific steatosis of the liver. This was substantiated by looking at a control BalbC liver. Therefore, we have qualified our pathology results by referring to data as "normal for the given strain" throughout the body of this document. Additionally, it is normal for mice in general to have spleens with extramedullary hematopoiesis.

In summary, the preclinical studies demonstrated that no significant toxicity could be identified following procedures that are planned in human cancer patients. Even though the histologic changes seen in the liver appear to be strain specific, baseline and post-treatment LFT's will be monitored to specifically identify any potential hepatic changes.

2.0 OBJECTIVES

Specific Aim: The safety of IV administration of phage RPLs in human patients with breast cancer will be established with both naïve libraries and amplified libraries. These studies will be the equivalent of Phase I trials. Screenings will be performed three times over a time period of ≤ 10 days. Throughout the screening process patients will be carefully evaluated for adverse reactions. Peptides displayed by phage isolated from tumor tissue will be routinely analyzed for both consensus amino acid sequences and tumor-binding. Binding to normal tissue will be assessed by immunohistology on a large panel of 35 different normal human tissues. Successful completion of this aim will establish the safety of in vivo phage RPL screening in humans and will result in the identification of peptides which bind specifically to breast tumor cells or to blood vessels specifically supplying tumor cells in human patients. Whole body in vivo screening experiments will result in the development of methods that may allow identification of novel tumor targets and greatly improved therapeutics.

Hypotheses 1. Serial panning of RPL's in human cancer patients over a 10 day time period will be nontoxic 2. Small peptides can be identified from RPLs that will bind specifically to human tumor cells and/or to blood vessels specifically supplying tumor by *in vivo* screening in human breast

cancer patients. 3. One way of identifying these peptides, and perhaps the most efficient way, is to inject phage RPLs libraries into patients and to harvest specific peptide-phage directly from resected tumor tissue.

3.0 PATIENT ELIGIBILITY CRITERIA

3.1 Histologic documentation: patients with metastatic or locally advanced breast cancer

3.2 Prior Treatment: No Limitations. No medications will exclude patients from this trial.

3.3 Measurable Disease:
Superficial cancer nodules or mass amenable to biopsy with minor surgery.

3.4 Age: ≥ 18 years of age
Performance score: Karnofsky status ≥ 70
Life expectancy: ≥ 4 months
Non-pregnant

3.5 Informed Consent: the patient must be aware of the nature of his/her disease and willingly consent after being informed of the procedure to be followed, the experimental nature of the procedure, alternatives, potential benefits, side effects, risks, and discomforts.

3.6 No concurrent malignancy is allowed

3.6 No other serious medical illness, other than that treated by this study, which would limit survival to < 4 months, or psychiatric condition which would prevent informed consent.

3.8 Organ Function:

Hematologic: Hgb ≥ 10 gm%, Hct ≥ 30%, ANC 1500/ul, Platelets ≥ 75,000/ul
Renal: Creatinine < institutional upper limit of normal
Hepatic: Less than 2 x upper limit of normal;

Albumin 3 – 5.5
Alk Phos 38 – 126
ALT 15 – 75
AST 8 – 50
Direct Bili 0.0 – 0.3
Total Bili 0.2 – 1.3
Total Protein 6 – 8.5

Cardiac: NYHA Grade II or less
Pulmonary: No evidence of impaired lung function on physical examination; if evidence of pulmonary metastases or history of COPD or other pulmonary problem prior to enrollment must have FEV, and/or Dfco ≥ 60% for enrollment.
Neuro: No clinical symptoms suggestive of brain metastases unless ruled out by imaging studies.

4.0 Patient Registration

Patients will be identified by participating physicians. Eligible patients will be approached by their physician about participating in the trial. The patient will be given the consent form to

read and an opportunity to ask any questions. The participating physician will be personally involved in the consent process. Patients will be encouraged to speak to the ombudsman assigned to this protocol. The role of the ombudsman is to provide patients with an impartial and independent point of view. They can also help raise concerns patients may have and see that they are addressed. The ombudsman will be available to speak with patients in person and on the phone throughout their participation in this study. Patients will be provided the name and contact information for the ombudsman assigned to this protocol.

If the patient chooses to participate, they will be asked to sign the consent form. The patient will be given a copy of the consent form.

Data sheets on all volunteers participating in this research will be submitted for entry into the U.S. Army Medical Research and Materiel Command (USAMRMC) Volunteer Registry Data Base. The information to be entered into this confidential data base includes the patients name, address, Social Security number, study name and dates. The intent of the data base is two-fold; first, to readily answer questions concerning an individual's participation in research sponsored by USAMRMC; and second, to ensure volunteers are adequately warned of risks and to provide new information as it becomes available.

4.1 Authorized physicians or designees must fill out confirmation of registration sheet (Appendix II) fax into the Operations Center 656-1987 to obtain a patient number. Office hours are 8:00 to 4:30.

4.2 All patients will sign an approved informed consent that provides full disclosure of the procedure, rationale, plan, and risks.

4.3 At the time of registration, the investigator may be asked to respond to a list of questions related to the patient's eligibility for this protocol. The eligibility checklist is located in Appendix II

4.4 When a patient is removed from protocol (e.g., because of disease progression or drug-related toxicity), the Operations Center is to be called and given the reason for the patient's removal from the protocol and the date on which discontinuation of protocol occurred.

4.5 We estimate that we will study 20 patients.

5.0 Required Monitoring of Patients

Patients will be admitted to the General Clinical Research Center (GCRC). An intravenous line will be placed. Baseline vital signs (blood pressure, pulse, temperature, and respiratory rate) will be determined before infusion of phage, every 15 minutes during infusion and for 2 hours after infusion. The patient will be under constant supervision and equipment, medications, and personnel capable of treating allergic (including anaphylactic reactions) will be immediately available. Blood (20ml) will be drawn prior to first infusion to determine eligibility and establish a baseline. Blood will be drawn at one month (10ml) and 6 weeks to 2 months (10ml) after the last infusion to evaluate patients for delayed toxicities and to prepare for proposed PCR testing. While we do not have a PCR technique developed and available for use at this time, the blood drawn 6 weeks to 2 months following the last phage injection will be stored at -20°C for future PCR testing should this be judged necessary.

	Before Infusion	Every 15 Minutes During Infusion	Two Hours after Infusion	One Month After Last Infusion	6 Weeks – 2 Months after Last Infusion
Blood Pressure	X	X	X	X	X
Pulse	X	X	X	X	X
Temperature	X	X	X	X	X
Respiratory Rate	X	X	X	X	X
Blood Draw	X			X	X

Screening will be completed in less than 10 days to avoid patient Ab response to phage [62]. Throughout the screening process patients will be carefully evaluated for adverse reactions.

6.0 Study Plan

The members of the research team include:

Principal Investigator: Responsible for all aspects of the protocol. This will include providing informed consent, establishing eligibility status, clinical monitoring, and performance of biopsies. Backup surgical and clinical coverage is provided continuously through the Division of Surgical Oncology.

Data Manager: Responsible for overseeing the recruitment and consent process, data collection and division of responsibilities related to this protocol.

GCRC nurse: Responsible for admitting patient to research ward, starting intravenous line, obtaining and recording vital signs, assisting surgeon with biopsy procedure, and drawing blood sample as needed.

Research Technologist: Responsible for preparation of infusion material. This includes all aspects of phage library preparation, sterilization, record keeping, and harvesting of phage.

Pathologist: Responsible for review and staining of all pathological specimens.

Approximately 20 patients will undergo in vivo screening in an attempt to identify peptides that home specifically to their tumor tissue. It is highly unlikely that cancer patients have preexisting intact filamentous phage located in their tumor tissue. In the experimental group, if phage clones isolated from tumor tissue after the third tissue harvest display a consensus amino acid sequence, it is highly likely that those peptide-phage are binding specifically to some component of the patient's tissue. An internal control will be to compare the sequences of phage clones eluted from the first tissue harvested (expected to be relatively random) compared to the last tissue harvested, as well as comparing clone sequences from peptide-phage isolated from different patients. Regarding the ability of this procedure to identify peptides which home specifically to tumors, we do not believe methods to minimize bias on the part of subjects, investigators, and analysis are necessary, as the measurements to be made: the number of phage eluted from the tumor, and the sequence of the peptides displayed by phage clones eluted from tumor, are objective and not subject to human bias. Regarding the possible side effects caused by this procedure, bias will be minimized by having all procedures performed in the University of Vermont General Clinical Research Center (GCRC). The staff at the GCRC are expert in observation of patients during experimental protocols and are not supervised by the PI of this investigation.

Patients will undergo pre-screening tests to confirm eligibility. Blood (volume 20 ml) will be drawn to measure hematologic, renal, and hepatic function. A blood test will be required to determine that a woman of child bearing age is not pregnant prior to participation in this study.

Women with a documented history or status (hysterectomy) that excludes the possibility of becoming pregnant will not require the pregnancy test. History and physical exam will include methods for determining whether a patient has pulmonary impairment or signs or symptoms of brain metastases. Pulmonary function studies and/or brain MRI or CT scan will be performed to confirm or rule out any suspicious clinical findings. Eligible patients will be given a premedication plan (prescriptions and instructions) prior to the first procedure to decrease the risk of an allergic reaction.

The risk of infection is present during any type of injection. There might also be slight discomfort at the injection site. If a contrast agent is used (for a scan) there is a small risk of allergic reaction. Patients will be advised to avoid becoming pregnant for at least one month after participation in this study. To avoid becoming pregnant, the patient should either abstain from sexual relations or practice a method of birth control. Except for surgical removal of the uterus, birth control methods such as the use of condoms, a diaphragm or cervical cap, birth control pills, IUD, or sperm killing products are not totally effective in preventing pregnancy.

Patients will be admitted to the GCRC. An intravenous line will be placed. Baseline vital signs (blood pressure, pulse, temperature, and respiratory rate) will be determined before infusion of phage and every 15 minutes during infusion and for 2 hours after infusion. The patient will be under constant supervision and equipment, medications, and personnel capable of treating allergic (including anaphylactic reactions) will be immediately available. Based on previous studies [13, 36, 58, 62] very few individuals have pre-existing bacteriophage antibodies and it is expected that antibacteriophage antibodies will develop subsequent to phage library administration similar to that demonstrated for ϕX174.

Ideally, we will screen our RPL(s) three times in the same patient. In the unlikely event of toxicity with naïve libraries, no further pans will be performed. In the event that only amplified phage show evidence of toxicity, no further pans will be performed.

A phage displayed RPL pool will be prepared and tested according to FDA standards as discussed below in "Pharmaceutical Information/Preparation". Before phage injection, one sample of tumor tissue from the patient will be biopsied, snap frozen and stored for later testing of selected clones for tumor binding affinity. A small portion of the tissue sample will be fixed in formalin and submitted for histological analysis and confirmation that harvested tissue is tumor tissue.

The library will be diluted in 100 - 250 ml saline and infused intravenously over approximately 10 minutes into a breast cancer patient. Collection of tumor-bound phage will performed by obtaining small amounts of tumor tissue. Tumor-tissue acquisition (biopsy) will occur in the procedure room of the GCRC approximately 10 – 60 minutes post infusion. Standard surgical technique for performing a surgical biopsy will be utilized for the biopsy procedures. It is intended that the biopsies be as small as possible and will be on the order of 1.0 gram of tissue. The method of biopsy will be either incisional, excisional, or core depending on the location of the tumor.

The tumor will be rinsed to remove blood, the tissue will be ground, and *E. coli* will be added to amplify phage. A small portion of the tumor will be fixed in formalin and submitted for histological analysis to confirm that harvested tissue is tumor tissue. Phage will be eluted from tumor cells and amplified using methods established by us and others [2, 5, 25, 61]. Phage will be amplified and quantified by titering, with results available within 12 - 24 hours. Enriched phage will be prepared as was described above for initial infusion and will be reinjected as soon as possible (1 to 2 days). Phage administration, tumor harvest, and amplification will be repeated a maximum of 2 times for a maximum of three screenings in one patient. Screening will completed within 10 days and ideally in less than 7 days to avoid patient Ab response to phage [62].

The primary end points for dose escalation will be toxicity (stopping) and phage isolation from tumor (continuing). The definition of successful phage isolation will be 1) the presence of phage in the tumor after each tumor tissue harvest, and 2) the presence of at least one three-amino acid consensus sequence in clones from the final pan.

The first patient will receive 10^{9-10} phage, and phage isolation from tumor will be evaluated. If no phages are isolated from the tumor, then a second patient will be treated with 10^{11} phages and attempt isolation from tumor. If the dose of phage must be escalated to 10^{12} or greater, three patients will be treated at each dose level to assure safety of the intravenous injection of that concentration of phage. Increase in the phage concentration will not be $> 10^{1}$ at any step in the dose escalation as based on titering. Once an acceptable amount of phage is observed in the tumor specimen, amplification and readministration of phage to patients will be initiated with each patient to receive up to three injections over a period of approximately seven days. The concentration of phage used for reinfusion will not exceed the initial concentration used for that patient unless safety of the higher dose of phage infusion has been demonstrated previously in three patients.

Phage will be amplified from the final tumor harvest. At least 20 clones will be selected and sequenced. If all patients at that phage dose level have consensus sequences (indicating sufficient copy number of phage at time of initial phage administration) no further dose escalation will be performed (see section 12: Statistical Considerations).

Throughout the screening process patients will be carefully evaluated for adverse reactions. Injection of phage will be stopped immediately in any patient if Grade II or greater allergic reactions (NCI CTC Version 2.0) occur. Further doses of phage will not be administered to that patient. Other NCI-CTC toxicities grade III or greater at any dose level are grounds for discontinuation of phage dose and for escalation of phage dose. Patients will be evaluated approximately one month after completion of phage injections(s) for delayed toxicities.

Peptides displayed by phage isolated from tumor tissue will be routinely analyzed for both consensus amino acid sequences and tumor-binding. Clones that bind to tumor tissue will be assessed for binding to non-tumor tissue. A panel of different non-tumor tissues will be obtained from the Vermont Cancer Center Tissue Procurement Facility. Immunohisotochemistry with anti-transferring receptor mAb will be used as a positive control to assure tissue and assay reliability.

Any consensus sequences identified from phage eluted specifically from the tumor tissue will be excellent candidates for tumor-specific peptides. Promising peptides will be synthesized and tested for tumor specificity. Peptide binders we identify by whole body screens, almost by definition, are likely to be stable in serum and generally stable *in vivo*, a major advantage to this technique.

In future studies, we will evaluate the efficacy of peptide-therapeutic conjugates. In addition to attaching standard cytotoxic drugs to the ligands, in separate experiments we will attach immunogenic peptides, perhaps one to which most people have already been immunized against. The binding of a molecule bearing an immunogenic peptide to the tumor cell surface may stimulate the immune system to eliminate the tumor cell. Thus, instead of using dangerous chemicals or radiation, we may be able to direct the body's own immune system to more naturally eradicate tumor cells.

7.0 Pharmaceutical Information

Qualified personnel who are familiar with procedures that minimize undue exposure to themselves and to the environment will undertake the preparation, handling, and safe disposal of agents in a self-contained, protective environment.

Drug Information And Preparation:

Filamentous peptide-phage are prepared from *E. coli* cultures grown overnight on 2xYT media agar plates. The phage particles are resuspended in phosphate buffered saline with or without Trasylol (PBS +/- Trasylol) by "sweeping" the agar with an angled glass rod. The phage suspension is centrifuged twice to remove bacterial cells and filtered with a 0.22 um polyethersulfone membrane to completely remove any remaining *E. coli* cells. The phage are concentrated by precipitation with polyethylene glycol (PEG). The resulting pellet is resuspended in fresh PBS +/- Trasylol and the phage suspension is passed through a 0.45 μm cellulose acetate

filter. Endotoxins are removed from the preparation by performing three 1% (v/v) Triton X-114 extractions. The phage are concentrated with PEG again and the resulting pellet is resuspended in PBS +/- Trasylol. The phage suspension is shaken 10 min at 200 rpm on ice, followed by centrifugation. The supernatant containing the peptide-phage is passed through a 0.45 μm cellulose acetate filter, followed by passage through a pyrogen-free 0.2 μm cellulose acetate filter to sterilize the preparation.

According to FDA guidelines, establishment of the sterility of any preparation to be injected into humans must be performed by inoculation of the product into Fluid Thioglycollate Media and Tryptic Soy Broth. We have performed these sterility tests exactly as described in the Code of Federal Regulations (21CFR610.12) on representative preparations. These tests have confirmed the sterility of our preparations, as expected after filtration through a pyrogen-free 0.2 μm cellulose acetate filter.

The FDA guidelines for asserting sterility take 14 days. Although this 14 day waiting period is not compatible with the screening experiments we have proposed for the 2 reasons listed below, we will still perform the tests. In the event of a sterility test failure, investigation of the protocol, additional patient examinations and appropriate measures will be taken.

A) In our experience and judgement, when performing phage-display RPL screening, it is optimal to prepare a fresh batch of a phage-displayed library to optimize complexity of libraries, as some of the displayed peptides within the library may be more susceptible to degradation than other peptides. Ideally we would like to be able to establish proof of sterility, lack of endotoxins, and any other parameters necessary, within a few hours. It is possible that some of these difficulties may be avoided by freezing peptide-phage preparations. However, the freezing process may also compromise the stability of the peptides and therefore, the complexity of the libraries. It is possible that all the peptides which may bind to tumor targets may be stable for 14 days. This may be elucidated as the study progresses. Furthermore, freezing or other storage methods which would avoid peptide degradation for 14 days, will not address the problem raised in point B below. It may be possible to establish a large batch of libraries used for *initial* screenings only if a storage method which allows the displayed peptides to retain their tumor binding activity can be developed.

B) To perform 3 screens in one person, the screens will probably need to be performed in less than 10 days, in order to avoid rejection of phage by the immune system, as a detectable IgM response to injected phage typically begins to develop in humans in about 7 days. Serial screening in one person may be the most optimal way to identify tumor-homing peptides, and may well be the only way to identify tumor-specific targets which are individual to a given patient. Therefore, to perform three screens in one patient, a 14 day waiting period for results of sterility testing will not be compatible with the current protocol.

Administration: Peripheral intravenous line

8.0 Potential Toxicity, Dose Modifications, and Management

There is the risk of allergic reaction (including anaphylactic) associated with the administration of the phage library. The likelihood of this is very low since a similar (but different strain) of bacteriophage has been injected into thousands of patients with no serious sequelae. Premedication will be administered to minimize the risk and possible response of allergic reaction and will be administered before the phage infusion. This premedication protocol is similar to that used prior to administration of some chemotherapeutic medicines. Personnel skilled in handling allergic reactions will be immediately present during infusions and all equipment for handling such reactions (including anaphylactic) will be immediately available. A small number of individual molecules of each type of displayed peptide are not expected to have strong toxicity. Preliminary studies in mice performed in our laboratory have not identified toxicity related to serial panning.. Patients will be closely monitored. All necessary measures will be taken to counter any level of allergic reaction.

All reagents will be prepared according to FDA standards and tested for sterility and pyrogenicity prior to administration. There is an extremely low risk of a patient receiving material that is either pyrogenic or not sterile.

Dr. Hans Ochs, a collaborator on this project, has the world's largest experience injecting bacteriophage and is a collaborator on this proposal. He has performed extensive studies with bacteriophage injected IV in humans. It has been used safely in over 3000 patients to monitor antibody responses with only rare adverse reactions in patients with unusual genetic immune deficiencies. Investigations were carried out to detect phage DNA incorporation into eukaryotic cells and was found only transiently in lymphocytes. He has an IND with the FDA for intravenous administration of bacteriophage. He also has extensive experience with the immunological consequences of intravenous administration of bacteriophage.

If patients are injured as a direct result of participating in this research project, the patient will be provided medical care, at no cost, for that injury. The patient will not receive any injury compensation, only medical care. The patient should understand that this will not be a waiver or release of their legal rights. The patient will discuss this issue thoroughly with the principal investigator before enrolling in this study.

9.0 Criteria for Response Assessment

This is not a therapeutic trial; therefore the change in tumor size will not be assessed. There is no direct benefit to the patients participating in this trial.

10.0 Removal of Patients from Protocol

Toxicity such as anaphylaxis, other intolerable infusion related toxicities, or grade 3 or greater NCI-CTC toxicity (ies), other unexpected serious adverse events related to phage administration, patient refusal, or investigator decision due to a change in health status or noncompliance would be reasons for study discontinuation. In this event:

- Notify the study chair
- Document the reason(s) for withdrawal on flow sheets

11.0 Adverse Event Reporting and Reporting Requirements

All adverse events (AEs) occurring with any patient participating in this clinical trial will be reported to the Cancer Center Protocol Office as described below.

11.1 **Immediately (within 24 hours)** telephone the Protocol Office for any of the following reasons:

- Any and all serious and/or life-threatening events which may possibly be reasonably associated, i.e., may reasonably be regarded as caused by, or reasonably be regarded as probably or possibly caused by, the investigational drug used in this protocol, or due to drug administration.
- All fatal and unexpected events regardless of cause or association with study treatment.
- All first occurrence of any "unexpected" (previously unobserved or unreported) toxicity (regardless of Grade).

11.2 Definitions

The following definitions of terms as per Federal Regulations apply to this section:

- ***Serious adverse experience*** means any experience that suggests a significant hazard, contraindication, side effect, or precaution. With respect to human clinical experience, a serious adverse drug experience includes any adverse drug experience that is fatal or life-threatening, is permanently disabling, requires inpatient hospitalization, or is a congenital anomaly, cancer, or overdose.

- ***Associated with the use of the drug*** means there is reasonable possibility that the experience may have been caused by the drug or combination of drugs.

- ***Unexpected adverse experience*** means any adverse experience that is not identified in nature, severity, or frequency in the current investigator brochure; or, if an investigator brochure is not required, that is not identified in nature, severity, or frequency in the risk information described in the general investigational plan or elsewhere in the current application, as amended.

11.3 Algorithm for Reporting Adverse Reactions

- Any and all serious and/or fatal or life threatening events which may be associated with the investigational drug used in this protocol, or due to drug administration. (see above): Report by telephone or facsimile transmission within 24 hours regarding to the Cancer Center Protocol Office at 802-656-2967.

  Person to be contacted:

  VCC Records Coordinator
  2nd Floor Medical Alumni Building
  Burlington, VT 05405
  Phone: 802-656-2967
  Fax: 802-656-8788
  Email: elizabeth.mensch@zoo.uvm.edu

- Any and all serious and/or fatal or life threatening events which are not associated with the investigational drug used in this protocol or with drug administration should be reported to the Cancer Center Protocol Office within 5 days.

- A written report of all adverse effects or experiences and deaths will be submitted by the investigator/co-investigator. In this report, the investigator will advise whether or not the AE is judged to be attributable to the study medication. All such subjects should be followed clinically by the appropriate diagnostic studies. Side effect or subjective symptomatology volunteered by a subject will be noted and recorded as to type and severity on the individual's patient chart. If no side effects are experienced, this also will be reported on the patient chart.

- A Medical Monitor has been assigned to this study. Marie Wood, MD has agreed to serve as the Medical Monitor. Dr. Wood is a qualified physician, not associated with this particular protocol, and able to provide medical care to research subjects for conditions that may arise during the conduct of the study. Dr. Wood is required to review serious and unexpected adverse events associated with the protocol and provide an unbiased written report of the event to the USAMRMC Office of Regulatory Compliance and Quality within 10 calendar days of the initial report.

- In turn the Protocol Office will inform the University Institutional Review Board (IRB) and the VCC Protocol Review Committee (PRC) and the FDA if necessary.

11.4 Responsibilities Of The Principal Investigator To The Surgeon General Through The USAMRMC, Office Of Regulatory Compliance And Quality

1. To promptly report changes or unanticipated problems in a research activity. Normally, changes may not be initiated without TSG approval, except where necessary to eliminate apparent immediate hazards to the human subject or others.
2. To immediately report by telephone (DSN 343-2165 or 301-619-2165) (non-duty hours call 301-619-2165 and send information by facsimile to DSN 343-7803 or 301-619-7803) adverse experiences that are both serious and unexpected.* For those projects involving an Investigational New Drug (IND) application sponsored by TSG, a written report will follow the initial telephone call within 3 working days.
3. To promptly report any change of investigators.
4. To prepare, at a minimum, an annual progress report or final report in accordance with Title 21, Code of Federal Regulations, Part 312.33.
5. To immediately report by telephone (DSN 343-2165 or 301-619-2165) knowledge of a pending compliance inspection by the Food and Drug Administration (FDA) or other outside governmental agency concerning clinical investigation or research.

11.5 Toxicity Grading Scale

The NCI Common Toxicity Criteria grading scale will be used. The investigator should describe the relationship of the toxicity to the study drug by using the terms "definite", "possible", "probable" or "unlikely".

12.0 Statistical Considerations

Successful binding of ligand can be viewed as a dichotomous variable and this perspective will be taken relative to the examination of the data. Ligand binding will be examined and the percent of subjects in whom a ligand is identified will be initially quantified using exact 95% binomial confidence interval. A sample size of n = 20 subjects would give rise to a standard error of at most 9.7% for a binomial point estimate under the assumption that the actual binding success rate would be at the 75% or higher level. With a 75% successful binding level, it is anticipated that an expected value of 7.5 out of the first 10 subjects examined would be observed. Since a change in the phage library would be desired if the library were not producing a sufficient rate of binding, we will examine the data in a sequential fashion. The probability of observing four or fewer successful events out of n = 10 subjects equals 2% using a cumulative binomial distribution with individual trial probability of success of 75%. Thus, if four or fewer successful events are observed in the first ten subjects, we will shift to a new phage library. If five or more of the first ten subjects have ligands identified, the next ten subjects will be examined using the same phage library. Cross reactivity with other tissue types (total tissue types = 32) will be examined individually for each tissue type using contingency table methods with quantification of the level of cross reactivity using a 95% confidence interval for the odds ratios. Identification of the prevalence of common peptide motifs will be estimated using exact 95% binomial confidence intervals. It is anticipated that a 30% prevalence of a common motif will give rise to a point estimate with a standard error of about 9%.

13.0 Records to be Kept

Data on the tumor characteristics will be collected from the pathology reports, infusion data (amount, batch number) will be recorded, and the molecular consensus sequences of possible binding peptides will be documented.

Representatives of the U.S. Army Medical Research and Materiel Command are eligible to review research records as part of their responsibility to protect human subjects in research.

The Surgeon General's Human Subjects Research Review Board (HSRRB) and the Human Subjects Committee and the Protocol Review Committee and Clinical Research Center at the University of Vermont must approve any modifications to the protocol prior to implementation.

Records will be kept as to whether or not patients wish for their samples to be used in future research. The patient will be given the opportunity to participate in this research protocol, but refuse to allow their samples to be used in other research. In this event, the samples with be disposed of in a manner consistent with medical tissue waste.

The Principal Investigator is responsible for:

1. Promptly reporting changes or unanticipated problems in a research activity. Normally, changes may not be initiated without approval, except where necessary to eliminate apparent immediate hazards to the human subject or others.
2. Immediately reporting by telephone adverse experiences that are both serious and unexpected.
3. Promptly report any change of investigators.
4. Submit a report to the UVM Committee on Human Research and to the Acting Chair of the Surgeons General's HSRRB after each subject, and after the first subject, wait 30 days before enrolling the second subject.
5. Prepare an annual progress report or final report in accordance with Title 21, Code of Federal Regulations, Part 312.33.
6. Immediately report by telephone knowledge of a pending compliance inspection by the Food and Drug Administration (FDA) or other outside governmental agency concerning clinical investigation or research.

14.0 References

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15.0 Informed Consent

Protocol Title: In Vivo Selection of Ligands for Targeted Therapy: Breast Cancer

Protocol Chairman: David N. Krag, M.D.

Responsible Physician: _____

Sponsor: Department of Defense and the Vermont Cancer Center, University of Vermont

You are invited to participate in a research study conducted at the University of Vermont by Dr._____. Your participation in this study is voluntary. You should read the information below, and ask questions about anything you do not understand, before deciding whether or not to participate.

PURPOSE OF THE STUDY

The purpose of this study is to develop a method to substantially increase the dose of anticancer drugs directly to a cancer while decreasing the dose to normal body tissue. How this will be accomplished will be explained in the following few paragraphs and in the following section called "Study Design". It is important for you understand that this study you are participating in does not involve treatment with anticancer drugs. Since no anticancer drugs will be administered as part of this study, you will receive no therapeutic benefit from participation in this study. It is also important for you to understand that this procedure is entirely new and that you need to carefully review and fully understand the section on potential risks (page number 3 of this form) involved in this study.

Anticancer treatments for breast cancer lack the ability to directly target cancer cells. Specifically, anticancer drugs are injected intravenously (by IV) and distribute throughout the entire body. The drug does not direct itself to cancer cells. Therefore, the entire body is exposed to the same dose of toxic anticancer drugs. The side effects produced by toxic anticancer drugs on normal non-cancer cells is a limiting factor for further treatment. This means that the dose of drug given can only be so much. It may take considerably more drugs than the body can tolerate in order for enough drug to reach and destroy the breast cancer.

We propose to develop a small tag, which is a molecule called a peptide, to the anticancer drug. This special tag would allow the anticancer drug to be delivered and concentrated at breast cancer cells. In this way, the concentration of drug would be much higher at the cancer cells than normal non-cancer cells.

The purpose of this study is to develop the tag that will attach to the "address" of cancer cells. In a future study the targeting agent (tag) will be designed to deliver anticancer drugs directly to the address of the cancer cells. There is a remote possibility that if this study is successful you may be asked to consider participation in subsequent clinical studies designed to test the anticancer effectiveness of this technique.

STUDY DESIGN

In order to find a tag to cancer cells we propose to intravenously inject several billion different tags (or molecules). A small piece of the cancer is removed and the tags that have found their way to the cancer cells (out of the billions injected) and are able to stick at the address of cancer cells will be determined.

Attaching each of the tags to a special agent called a bacterial virus allows your doctor to find the special tag that sticks preferentially to the cancer cells. Your doctor is able to find the bacterial viruses and is then able to determine the exact nature (or type) of the tag that sticks to your cancer.

The bacterial viruses (which carry the tag) are living agents that are similar to the human viruses (or germs) that cause the flu and other minor and sometimes serious illnesses in people. These bacterial viruses are special and are not known to infect humans or any other animals. They are not known to cause any human illnesses. They are only able to infect a very small living cell called a bacterium. Several thousand humans have been previously injected with special bacterial viruses (similar, but not identical, to the ones proposed here) and adverse responses are extremely rare.

Following injection of the special tags, your doctor will remove a small piece of cancer from your body. The size of this piece of cancer will be a cube about 1/2 an inch on edge. The reason for removing this piece is to find the special tags that have stuck to your cancer cells. This procedure will be a minor surgical procedure and may involve a small incision and placement of stitches to close the wound. This procedure will be done under local anesthesia.

It is expected that your doctor will find many tags in the cancer specimen that was removed from your body. Some of these tags are there because they truly stick strongly to your cancer cells. These are the tags that your doctor is after. Some of the tags, however, will be just passersby. That is, some of the tags will just happen to be passing though the cancer at the time the small specimen is removed and will not really be sticking to the cancer.

In order to determine which tags truly stick and which do not (or do not stick very well) your doctor will purify the tags found in your cancer and later reinject them into your vein. Just as was done following the first injection, a piece of cancer will be removed. This time there will be many less types of special tags to bind to your cancer cells and it is more likely that your doctors will be able to identify the tags that stick most strongly to your cancer cells. The entire procedure of injection and biopsies will be repeated a third time. All of the injections and biopsies will be performed within 10 days. Each of these three visits (for the injection and biopsy) will take approximately 5 hours. You will be asked to return 1 and 2 months following your third biopsy. These visits will take approximately 30 minutes. Your vital signs will be taken, a blood sample of 10 ml (2 teaspoons) will be taken and a physical exam will be performed.

Before the first injection takes place your doctor will perform a biopsy to have enough of your cancer cells available for later testing. The tags later identified to stick most strongly to your cancer cells will be tested against the first cancer biopsy material to determine how sticky they are.

This entire procedure: 1) biopsy cancer tissue, 2) injection of special tags in your veins and biopsy of cancer tissue, and 3) repeat injection of special tags and repeat biopsy of cancer tissue, is only one set of important steps required before this method of targeting anticancer drugs can be useful in possibly treating cancer. This clinical study is only designed to determine if this method (injection of tags and biopsy of cancer tissue) can find the special tags that stick preferentially to your cancer cells.

In order for this method to be possibly useful in treating your cancer an entire additional set of studies need to be performed. These additional studies involve connecting the special tag to anticancer drugs for special delivery to your cancer cells. It is important for you to understand that these second set of studies will not be performed as part of this clinical study you are being invited to participate in. That means that the findings from this clinical study will likely not be useful to you personally in treating your cancer. You will receive no actual treatment for your cancer as part of this study.

Twenty people will be enrolled in this study.

POTENTIAL RISKS AND DISCOMFORTS

There is a potential for unforeseen risks/complications. While similar agents to the one being tested have been used in humans with minimal serious problems, you will be one of the first humans to receive this specific agent. This investigational agent has been tested in animal studies designed to mimic the proposed human studies. The agent to be used in this study is not approved by the Food and Drug Administration (FDA) for commercial use. However, based on the success of the animal studies, the FDA permitted its use in this research study. While serious complications are not expected, it is impossible to guarantee that none will occur.

You will be given a premedication plan (prescriptions and instructions) prior to the first procedure to decrease the risk of an allergic reaction. You will be monitored very closely by either a nurse, or your doctor, or both, during the time of the injection of the material into your veins. There is a risk of allergic reaction to the material. An allergic reaction may result in hives and itching or a more severe reaction could result in shortness of breath and lowering of blood pressure. Although the risk of a severe reaction is very low (less than 1 in a thousand) it is real and may be serious and possibly life threatening. Medications will be immediately available to counteract any allergic reactions, no matter how severe.

You will be asked to take a blood test to determine pregnancy status if you are a woman and of child bearing age, unless you have a documented history or status (hysterectomy) that excludes the possibility of pregnancy. You will be advised to abstain from sexual relations or practice a method of birth control during the time of your participation in the study and for one month following the study. Since we do not know the risk the material poses to a fetus, pregnant women are excluded from this study.

When you have blood drawn there is a small risk of infection, a minor amount of discomfort typical for drawing of blood and the possibility of bruising.

An intravenous catheter will be placed into one of your veins. This is called an "IV" and it may be uncomfortable since it requires placement of a needle into your vein first. It will remain in your arm for several hours until the entire procedure is complete. A risk of having an intravenous catheter placed is an infection. This is a small risk but is real. If this should happen you may need to put warm soaks on the area and may need to take an antibiotic. There is also a risk of bleeding, swelling or bruising.

The material you will be injected with will be put through a filter which blocks out bacteria. Although this filter is used and all precautions are taken to keep the material sterile, there is a small risk of infection. If this should happen you may need to take an antibiotic.

You will have up to four biopsies of your cancer tissue. Each one of these biopsies will be small but will involve a small surgical procedure. Local anesthesia will be injected around the biopsy site to numb the tissue. You may need to have stitches placed. The biopsy sites will have a dressing. This will need to remain in place for about 48hrs. Following that the dressing should be removed and the area gently cleansed with soap and water (taking a shower will be fine). There is a small risk of infection as there is with any surgery. If the area becomes reddened, swollen, or develops increasing pain it should be evaluated by a doctor that same day and you may need to take an antibiotic. There should be little restrictions on activity and you should avoid rigorous physical activities that would cause discomfort to the biopsy site. You should be able to perform your normal activities within 1 to 2 days. A prescription for pain medication will be provided although it is expected that the discomfort will be relatively minimal.

You should report to your doctor if you get a rash, sore joints, or itching. This could be a sign of a delayed allergic reaction. These symptoms usually occur between 2 to 7 days after the injection. If this occurs, your doctor will check your liver function and consider treatment with a short course of steroids.

ANTICIPATED BENEFITS TO SUBJECTS

This clinical study will be of no immediate or direct benefit to you. It may in the future lead to important findings that may benefit others.

ALTERNATIVES TO PARTICIPATION

Your therapy will not be lessened by participation in this trial. This clinical study is not a therapeutic study and therefore not related to your treatments.

PAYMENT FOR PARTICIPATION

You will not be responsible for any of the study related costs. Other medications and all physicians' and hospital costs related to your regular treatments will be charged to you in the same fashion as if you were not part of this study. You will receive no monetary compensation for your participation in this study.

POSSIBLE COMMERCIAL PRODUCTS

During this study, you will be asked to provide four biopsies of your cancer. These samples will be used for identifying the tags which bind to the cancer cells. There is a chance that the samples that you are donating under this study may be used in other research studies and may have some commercial value. You will be given the opportunity to participate in this research protocol, but may refuse to allow your samples to be used in other research. You will not receive any compensation for any future value that the sample you have given may be found to have. You will not receive any notice of future use of your samples.

MEDICAL CARE FOR RESEARCH RELATED INJURY

If you are injured as a direct result of participating in this research project, you will be provided medical care, at no cost to you, for that injury. You will not receive any injury compensation, only medical care. This is not a waiver or release of your legal rights. You should discuss this issue with the principal investigator before enrolling in this study.

CONFIDENTIALITY

A record of your progress will be kept in a confidential form at the Vermont Cancer Center, University of Vermont (VCC/UVM). The results of this study may eventually be published and that information may be exchanged between medical investigators, but patient confidentiality will be maintained. There is a possibility that your medical record, including identifying information, may be inspected and/or photocopied by qualified representatives from VCC/UVM, Fletcher Allen Health Care, the National Cancer Institute, the U.S. Army Medical Research and Materiel Command, Food and Drug Administration or other Federal or state government agencies in the ordinary course of carrying out their governmental functions. If your record is used or disseminated for government purposes, it will be done under conditions that will protect your privacy to the fullest extent possible consistent with laws relating to public disclosure of information and the law-enforcement responsibilities of the agency.

PARTICIPATON AND WITHDRAWAL

Your participation in this research is voluntary. If you choose not to participate, it will not affect your relationship with the Vermont Cancer Center or Fletcher Allen Health Care or your right to health care or

other services to which you are otherwise entitled. If you decide to participate, you are free to withdraw your consent and discontinue participation at any time without prejudice.

CONSEQUENCES OF WITHDRAWAL

If you choose to withdraw from this study, the principal investigator may request that you return for some follow up visits. If you also choose to withdraw your consent for any donated tissue, the tissue will be disposed of in a manner consistent with and appropriate for medical waste.

WITHDRAWAL OF PARTICIPATON BY THE INVESTIGATOR

The investigator may withdraw you from participating in this research if circumstances arise which warrant doing so. If you experience a severe allergic reaction or if you become ill during the research, you may have to drop out, even if you would like to continue. The investigator will make the decision and let you know if it is not possible for you to continue. The decision may be made either to protect your health and safety, or because it is part of the research plan that people who develop certain conditions may not continue to participate.

NEW FINDINGS

During the course of the study, you will be informed of any significant new findings (either good or bad), such as changes in the risks or benefits resulting from participation in the research or new alternatives to participation, that might cause you to change your mind about continuing the study. If new information is provided to you, your consent to continue participation in this study will be re-obtained.

IDENTIFICATION OF INVESTIGATORS

In the event of a research related injury or if you experience an adverse reaction, please immediately contact the investigator listed below. If you have any questions about the research, please feel free to contact Dr. David Krag, One South Prospect St. (802-847-2262).

VOLUNTEER REGISTRY DATA BASE REQUIREMENTS

Data sheets on all volunteers participating in this research will be submitted for entry into the U.S. Army Medical Research and Materiel Command (USAMRMC) Volunteer Registry Data Base. The information to be entered into this confidential data base is limited to your name, address, Social Security number, study name and dates. The intent of the data base is two-fold; first, to readily answer questions concerning your participation in research sponsored by USAMRMC; and second, to ensure that you are adequately warned of risks and to provide new information as it becomes available.

RIGHTS OF RESEARCH SUBJECTS

You may withdraw your consent at any time and discontinue participation without penalty. You are not waiving any legal claims, rights or remedies because of your participation in this research study. If you have questions regarding your right as a research subject, you may contact Nancy Stalnaker, the Institutional Review Board Administrator, 231 Rowell Building (802-656-4067).

You are encouraged to speak to the ombudsman assigned to this protocol. The role of the ombudsman is to provide you with an impartial and independent point of view. They can also help raise concerns you might have and see that they are addressed. The ombudsman will be available to speak with you in person and on the phone throughout your participation in this study. You will be provided the name and contact information for the ombudsman assigned to this protocol.

SIGNATURE OF RESEARCH SUBJECT

I have read the information provided above. I have been given an opportunity to ask questions and all of my questions have been answered to my satisfaction. I understand that I may ask further questions and that I may withdraw from the study at any time. I agree to participate in this study and I understand that I will be given a copy of this consent form.

_____
Name of Patient

_____ _____
Signature of Patient Date

_____
Address

SIGNATURE OF WITNESS

My signature as witness certifies that the subject signed this consent form in my presence as his/her voluntary act and deed.

_____
Name of Witness

_____ _____
Signature of Witness Date

SIGNATURE OF PERSON OBTAINING CONSENT

_____
Name of Person Obtaining Consent

_____ _____
Signature of Person Obtaining Consent Date

Principal Investigator:

David Krag, M.D.
Surgical Associates
One South Prospect
Burlington, VT, 05401
802-847-2262

SAMPLE DONATION FORM

Protocol Title: In Vivo Selection of Ligands for Targeted Therapy: Breast Cancer

Protocol Chairman: David N. Krag, M.D.

Sponsor: Department of Defense and the Vermont Cancer Center, University of Vermont

As a participant in "In Vivo Selection of Ligands for Targeted Therapy" protocol, I voluntarily donate my cancer tissue removed during the four biopsies to the University of Vermont. These samples will be used for identification of tags (or molecules) which find their way to my cancer and for uses not currently known to me. There is a possibility that the samples that I am donating under this study may be used in other research studies and may have some commercial value. Should my donated samples lead to the development of a commercial product, the University of Vermont will own it and it is possible that it will be patented and licensed by the University of Vermont. The University of Vermont does not intend to provide me any compensation for this and will not give me any notice of future uses of my samples.

I have read the information provided above. I have been given an opportunity to ask questions and all of my questions have been answered to my satisfaction.

SIGNATURE OF PATIENT

_____
Name of Patient

_____ _____
Signature of Patient Date

_____
Address

SIGNATURE OF WITNESS

My signature as witness certifies that the subject signed this consent form in my presence as his/her voluntary act and deed.

_____
Name of Witness

_____ _____
Signature of Witness Date

Principal Investigator:
David Krag, M.D., Surgical Associates, One South Prospect, Burlington, VT, 05401
802-847-2262

APPENDIX I

NCI COMMON TOXICITY CRITERIA

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life-Threatening)
BLOOD/BONE MARROW					
WBC	≥ 4.0	3.0 - 3.9	2.0 - 2.9	1.0 - 1.9	< 1.0
PLT	WNL	75.0 - normal	50.0 - 74.9	25.0 - 49.9	< 25.0
Hgb	WNL	10.0 - normal	8.0 - 10.0	6.5 - 7.9	< 6.5
Granulocytes/ Bands	≥ 2.0	1.5 - 1.9	1.0 - 1.4	0.5 - 0.9	< 0.5
Lymphocytes	≥ 2.0	1.5 - 1.9	1.0 - 1.4	0.5 - 0.9	< 0.5
LIVER					
Bilirubin	WNL	–	< 1.5 x N	1.5 - 3.0 x N	> 3.0 x N
Transaminase (SGOT, SGPT)	WNL	≤ 2.5 x N	2.6 - 5.0 x N	5.1 - 20.0 x N	> 20.0 x N
Alk Phos or 5' nucleotidase	WNL	≤ 2.5 x N	2.6 - 5.0 x N	5.1 - 20.0 x N	> 20.0 x N
GASTROINTESTINAL					
Nausea	none	able to eat reasonable intake	intake significantly decreased, but can eat	no significant intake	—
Vomiting	none	1 episode in 24 hours	2-5 episodes in 24 hours	6-10 episodes in 24 hours	> 10 episodes in 24 hours or requiring parenteral support
Diarrhea	none	increase of 2-3 stools/day over pre-Rx	increase of 4-6 stools/day, or nocturnal stools, or moderate cramping	increase of 7-9 stools/day, or incontinence, or severe cramping	increase of ≥10 stools/day, or grossly bloody diarrhea, or need for parenteral support
Stomatitis	none	painless ulcers, erythema, or mild soreness	painful erythema, edema, or ulcers, but can eat	painful erythema, edema, or ulcers, and cannot eat	requires parenteral or enteral support
KIDNEY/BLADDER					
Creatinine	WNL	< 1.5 x N	1.5 - 3.0 x N	3.1 - 6.0 x N	> 6.0 x N
Proteinuria	no change	1+ or <0.3 g% or < 3 g/l	2 - 3+ or 0.3 - 1.0 g% or 3 -10 g/l	4+ or > 1.0 g% or > 10 g/l	nephrotic syndrome
Hematuria	neg	micro only	gross, no clots	gross + clots	requires transfusion

APPENDIX I (Continued)

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life-Threatening)
HEART/LUNGS					
Cardiac dysrhythmias	none	asymptomatic, transient, requiring no therapy	recurrent or persistent, no therapy required	requires treatment	requires monitoring; or hypotension, or ventricular tachycardia, or fibrillation
Cardiac function	none	asymptomatic, decline of resting ejection fraction by less than 20% of baseline value	asymptomatic, decline of resting ejection fraction by more than 20% of baseline value	mild CHF, responsive to therapy	severe or refractory CHF
Cardiac–ischemia	none	non-specific T-wave flattening	asymptomatic, ST and T wave changes suggesting ischemia	angina without evidence for infarction	acute myocardial infarction
Cardiac–pericardial	none	asymptomatic effusion, no intervention required	pericarditis (rub, chest pain, ECG changes)	symptomatic effusion; drainage required	tamponade; drainage urgently required
Pulmonary	none or no change	asymptomatic, with abnormality in PFT's	dyspnea on significant exertion	dyspnea at normal level of activity	dyspnea at rest
Weight gain/loss	< 5.0%	5.0 - 9.9%	10.0 - 19.9%	≥ 20.0%	—
BLOOD PRESSURE					
Hypertension	none or no change	asymptomatic, transient increase by greater than 20 mm Hg (D) or to > 150/100 if previously WNL; no treatment required	recurrent or persistent increase by greater than 20 mm Hg (D) or to > 150/100 if previously WNL; no treatment required	requires therapy	hypertensive crisis
Hypotension	none or no change	changes requiring no therapy (including transient orthostatic hypotension)	requires fluid replacement or other therapy	requires therapy and resolves within 48 hours of stopping the agent	requires therapy for > 48 hours after stopping the agent

APPENDIX I (Continued)

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life Threatening)
NEUROLOGIC					
Neuro--sensory	none or no change	mild paresthesias, loss of deep tendon reflexes	mild or moderate objective sensory loss; moderate paresthesias	severe objective sensory loss or paresthesias that interfere with function	—
Neuro--motor	none or no change	subjective weakness; no objective findings	mild objective weakness without significant impairment of function	objective weakness with impairment of function	paralysis
Neuro--cortical	none	mild somnolence or agitation	moderate somnolence or agitation	severe somnolence, agitation, confusion, disorientation, or hallucinations	coma, seizures, toxic psychosis
Neuro--cerebellar	none	slight incoordination dysdiakokinesis	intention tremor, dysmetria, slurred speech, nystagmus	locomotor ataxia	cerebellar necrosis
Neuro--mood	no change	mild anxiety or depression	moderate anxiety or depression	severe anxiety or depression	suicidal ideation
Neuro--headache	none	mild	moderate or severe but transient	unrelenting and severe	—
Neuro--constipation	none or no change	mild	moderate	severe	ileus .> 96 hours
Neuro--hearing	none or no change	asymptomatic, hearing loss on audiometry only	tinnitus	hearing loss interfering with function but correctable with hearing aid	deafness not correctable
Neuro--vision	none or no change	—	—	symptomatic subtotal loss of vision	blindness

APPENDIX I (Continued)

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life Threatening)
DERMATOLOGIC					
Skin	none or no change	scatter macular or papular eruption or erythema that is asymptomatic	scatter macular or papular eruption or erythema with pruritus or other associated symptoms	generalized symptomatic macular, papular, or vesicular eruption	exfoliative dermatitis or ulcerating dermatitis
Palmar-Plantar Erythro-dysesthesia	no symptoms	Mild erythema, swelling, or desquamation not interfering with daily activities.	Erythema, desquamation, or swelling interfering with, but not precluding, normal physical activities; small blisters or ulcerations less than 2 cm in diam.	Blistering, ulceration, or swelling interfering with walking or normal daily activities; cannot wear regular clothing.	Diffuse or local process causing infectious complications, or a bed ridden state or hospitalization.
Allergy	none	transient rash, drug fever < 38°C, 100.4°F	urticaria, drug fever = 38°C, 100.4°F, mild bronchospasm	serum sickness, bronchospasm requiring parenteral medications	anaphylaxis
Alopecia	no loss	mild hair loss	pronounced or total hair loss	—	—
METABOLIC					
Hyperglycemia	< 116	116 - 160	161 - 250	251 - 500	> 500 or ketoacidosis
Hypoglycemia	> 64	55 - 64	40 - 54	30 -39	< 30
Amylase	WNL	< 1.5 x N	1.5 - 2.0 x N	2.1 - 5.0 x N	> 5.1 x N
Hypercalcemia	< 10.6	10.6 - 11.5	11.6 - 12.5	12.6 - 13.5	> 13.5
Hypocalcemia	> 8.4	8.4 - 7.8	7.7 - 7.0	6.9 - 6.1	≤ 6.0
Hypomagnesemia	> 1.4	1.4 - 1.2	1.1 - 0.9	0.8 - 0.6	≤ 0.5

APPENDIX I (Continued)

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life Threatening)
COAGULATION					
Fibrinogen	WNL	0.99-0.75 x N	0.74-0.50 x N	0.49-0.25 x N	≤ 0.24 x N
Prothrombin time	WNL	1.01-1.25 x N	1.26-1.50 x N	1.51-2.00 x N	> 2.00 x N
Partial thrombo-plastin time	WNL	1.01-1.66 x N	1.67-2.33 x N	2.34-3.00 x N	> 3.00 x N
Hemorrhage (Clinical)	none	mild, no transfusion	gross, 1-2 units transfusion per episode	gross, 3-4 units transfusion per episode	massive, >4 units transfusion per episode

Source (modified from): National Institute of Health, National Cancer Institute, Cancer Therapy Evaluation Program, Bethesda, Maryland 20892

Chills (rigors)	none	any rigor, mild	rigors requiring medication	rigors not controlled by medication	—

Appendix II

DATA COLLECTION FORMS

Registration & Eligibility Checklist
VCC Confirmation of Registration
Adverse Event Report
Patient Data Collection Form
Lab Data Collection Log Sheet

In Vivo Selection of Ligands for Targeted Therapy: Breast Cancer

Patient Registration Form (Page 1 of 3)

PLEASE PRINT

Surgeon Of Record: _____

Contact Person: _____

Phone Number: _____ FAX : _____

Patient Name: _____

Birth Date: ____/____/____ Date of Diagnosis: ____/____/____

Signed Consent Attached? ____ Yes ____ No

FAX BOTH REGISTRATION PAGES AND THE ENTIRE CONSENT FORM TO:
802-656-1987

PATIENT WILL NOT UNDERGO PROCEDURE UNLESS THIS SHEET IS FAXED BACK TO YOU WITHASSIGNED ID NUMBER.

BELOW LINE FOR OPERATIONS CENTER USE ONLY

Assigned Patient ID Number: _____

Date of Registration: ____/____/____

CHRMS #: _____

Registrar: _____

Checklist:

Registration form legible and complete ___ Consent form is correct for study ___
Consent form legible and complete ___ Consent form has not expired ___
Eligibility criteria are all checked "Y" ___

VCC registration form submitted ____

In Vivo Selection of Ligands for Targeted Therapy: Breast Cancer

Patient Registration Form (Page 2 of 3)

Eligibility Criteria

Patient Name: _____ Date of Birth: _____

1. The patient has metastatic or locally advanced primary or recurrent breast cancer — Y ___ N ___
2. The patient has superficial cancer nodules or mass amenable to biopsy with minor surgery. — Y ___ N ___
3. The patient has a Karnofsky status ≥ 70 and a life expectancy ≥ 4 months. — Y ___ N ___
4. The patient has undergone an informed consent process. — Y ___ N ___
5. The patient is not pregnant. — Y ___ N ___
6. The patient does not have any other serious illness, other than that treated by this study — Y ___ N ___
7. The patient ≥ 18 years of age. — Y ___ N ___
8. The patient has no evidence of extensive pulmonary metastases. — Y ___ N ___
9. The patient has no clinical symptoms suggestive of brain metastases. (unless ruled out by imaging) — Y ___ N ___
10. Hematologic: Hgb ≥ 10 gm%, Hct ≥ 30%, ANC 1500/ul, Platelets ≥ 75,000/ul — Y ___ N ___
11. Renal: Creatinine < institutional upper limit of normal — Y ___ N ___
12. Hepatic: Less than 2 x upper limit of normal; — Y ___ N ___

    Albumin 3 – 5.5
    Alk Phos 38 – 126
    ALT 15 – 75
    AST 8 – 50
    Direct Bili 0.0 – 0.3
    Total Bili 0.2 – 1.3
    Total Protein 6 – 8.5

In Vivo Selection of Ligands for Targeted Therapy: Breast Cancer

Patient Registration Form (Page 3 of 3)

13. Cardiac: NYHA Grade II or less Y ___ N ___

14. The patient has no other active cancer Y ___ N ___

All statements must be checked "Y" or "N/A" for entry into the trial.
Note: this sheet does not constitute source documentation. The above information must be included elsewhere in the patient's hospital chart in a recognized source document.

Signature of Physician: _____ Date: _____

VCC CONFIRMATION OF REGISTRATION

In Vivo Selection of Ligands for Targeted Therapy: Breast Cancer

Physician:_____

~~Patient Name:~~_____

(Please print) Last First Middle

Hospital Chart #:_____ Social Security # _____

Race: _____ Sex: Male Female Date of Birth ____/____/____

(1-White, 2-Hispanic, 3-Black, 4-Oriental, 5-Native Hawaiian, 6-Native American, 7-Indian, 8-Filipino, 9-Other, 10-Patient refusal, 1-Institution refusal, -1-unkown)

Method of Payment_____ Zip Code:_____

(1-Private Insurance,2-Medicare, 3-Medicare and private insurance, 4-Medicaid, 5-Medicaid and Medicare, 6-Military or Veterans Administration Sponsored, 7-Self Pay (no insurance), 8-No means of payment (no insurance), -1-unknown)

Eligibility Criteria:- See protocol checklist

_____Patient Eligible? (1-no; 2-yes, all requirements confirmed)

If different levels list here as 1,2 3.

Patient Study Number _____
Level Assigned _____
Date Registered_____
Registrar _____

REPORT OF ADVERSE EVENTS AND/OR UNANTICIPATED PROBLEMS

All items on this form must be completed by the principal/co-investigator. Please attach any additional information.

Submit completed form to:

Cancer Center Protocol Office
2nd Floor Medical Alumni Building
Burlington, VT 05405

FOR VCC USE ONLY
Date of Notification
Initial Contact ___/___/___
FU Contact ___/___/___

CHRMS #: _____ PRINCIPAL INVESTIGATOR: David N. Krag, MD

PROTOCOL NUMBER AND TITLE: In Vivo Selection of Ligands for Targeted Therapy

Patient Identification Number: _____ Date of event/problem: _____

Brief description of event/problem (please do not indicate "see attached" as a response):

Did event/problem occur here? Yes _____ No _____

Was event/problem related to protocol? Yes _____ No _____ Unsure _____
If yes, how?

Have there been similar events/problems reported here? Yes _____ No _____
Elsewhere? Yes _____ No _____
If yes to either, explain:

Was the protocol discontinued for this subject? Yes _____ No _____ Unknown _____

Was further treatment required? Yes _____ No _____
If yes, explain:

Does the protocol need to be modified as a result of this report? Yes _____ No _____
If yes, explain:

Does the consent form need to be modified as a result of this report? Yes _____ No _____
If yes, explain:

Note: You should keep a copy of this completed form as this information *must* be included in your summary of events/problems encountered during the indicated time period of *your next continuing* review.

PATIENT DATA COLLECTION FORM

In Vivo Selection of Ligands for Targeted Therapy: Breast Cancer

(Page 1 of 2)

Patient Name_____
MRN _____
DOB _____
Study ID Number _____
Date of first biopsy _____

Record of Vitals – First Infusion and Biopsy **Date:** _____

	Before Infusion	Every 15 Minutes During Infusion	Two Hours after Infusion
Blood Pressure			
Pulse			
Temperature			
Respiratory Rate			

Record of Vitals – Second Infusion and Biopsy **Date:** _____

	Before Infusion	Every 15 Minutes During Infusion	Two Hours after Infusion
Blood Pressure			
Pulse			
Temperature			
Respiratory Rate			

Record of Vitals – Third Infusion and Biopsy **Date:** _____

	Before Infusion	Every 15 Minutes During Infusion	Two Hours after Infusion
Blood Pressure			
Pulse			
Temperature			
Respiratory Rate			

Record of Vitals – Post Infusions

	One Month After Last Infusion **Date:** _____	6 wks – 2 mos after Last Infusion **Date:** _____
Blood Pressure		
Pulse		
Temperature		
Respiratory Rate		

PATIENT DATA COLLECTION FORM

In Vivo Selection of Ligands for Targeted Therapy: Breast Cancer

(Page 2 of 2)

Blood Work

Prior to infusion –	**Date:** _____	**Report #:** _____
One Month Post Infusion–	**Date:** _____	**Report #:** _____
6 wks – 2 months Post Infusion–	**Date:** _____	**Report #:** _____

LAB DATA COLLECTION LOG SHEET

In Vivo Selection of Ligands for Targeted Therapy: Breast Cancer

Study ID #	Date of peptide-phage infusion	Peptide-phage lot #	Peptide-phage infused (TU's)	Weight of tumor tissue for phage recovery (mg)	Peptide-phage recovered from tumor (TU/mg)	Passed 14-day sterility test? (yes/no)

The University of Vermont
COMMITTEES ON HUMAN RESEARCH
231 ROWELL
BURLINGTON, VERMONT 05405-0068
TEL. (802) 656-4067
FAX (802) 656-3190

PROTECTION OF HUMAN SUBJECTS ASSURANCE

Title: "In Vivo Selection of Ligands for Targeted Therapy: Breast Cancer"

Principal Investigator: David Krag, M.D.

Institution: University of Vermont and State Agricultural College
Burlington, VT 05405 802/656-4067

This institution has an approved assurance of compliance on file with the Department of Health and Human Services which covers this activity.

Assurance identification number M1375 IRB identification number 01XB

CERTIFICATION OF IRB REVIEW OR DECLARATION OF EXEMPTION

_X_ This activity has been reviewed and approved by an IRB in accordance with the requirements of 45 CFR 46, including its relevant Subparts. This certification fulfills, when applicable, requirements for certifying FDA status for each investigational new drug or device. The IRB classified the protocol as greater than minimal risk.

Date of approval FEB - 7 2001

___ This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by 45 CFR 46 will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

___ Human subjects are involved, but this activity qualifies for exemption under 46.101(b) in accordance with paragraph ___, but the institution did not designate that exemption on the application.

As a condition of approval, this institution's Committee on Human Research required ____ did not require _X_ changes and/or modifications to the above referenced application. (A list of required changes and/or modifications is attached as appropriate.) Continuing review of this protocol is due as follows: the Committee is requiring that a report be submitted after each subject, and that, after the first subject, the investigator wait 30 days before enrolling the second subject. Additional review conditions will be determined at that time.

Institutional Signature/Date: 2/20/01

Name and Title of Official: Ira Bernstein, M.D., Chair, Committee on Human Research in the Medical Sciences

CHRMS 01-170A

The University of Vermont

COMMITTEES ON HUMAN RESEARCH
231 ROWELL
BURLINGTON, VERMONT 05405-0068
TEL. (802) 656-4067
FAX (802) 656-3190

NOTIFICATION OF APPROVAL - AMENDED PROTOCOL/INFORMED CONSENT FORM

Title: "In Vivo Selection of Ligands for Targeted Therapy: Breast"

Principal Investigator: David Krag, M.D.

Sponsor Protocol Number:

Institution: University of Vermont and State Agricultural College
Burlington, VT 05405 802/656-4067

This institution has an approved assurance of compliance on file with the Department of Health and Human Services which covers this activity.

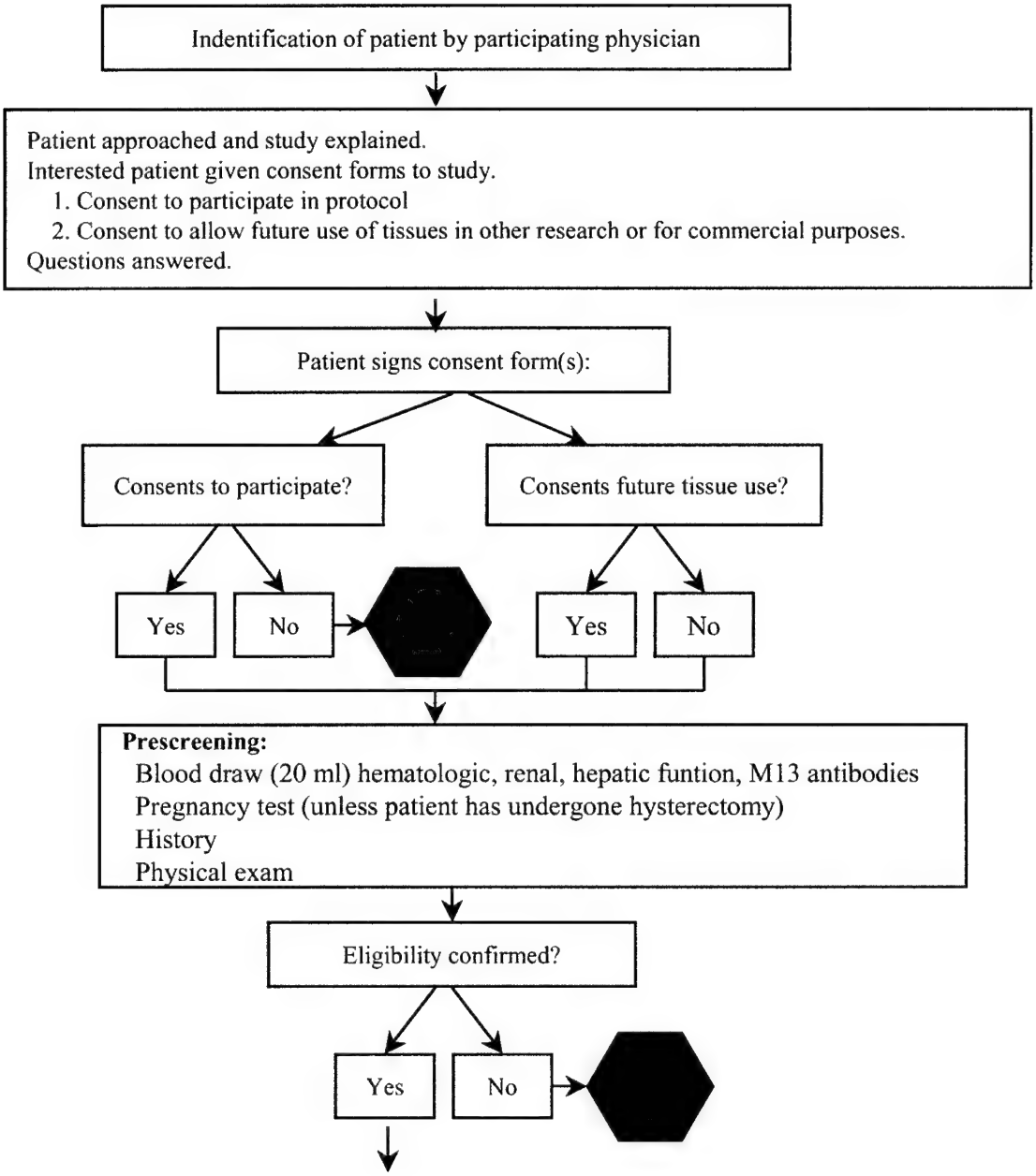
Assurance number for University of Vermont and State Agricultural College: FWA 723
IRB number: IRB 485
(Fletcher Allen Health Care Assurance number: FWA 727)

Items submitted for approval:		Date approved:
[X]	Protocol dated: 7/6/01	AUG 31 2001
[ ]	Amendment dated:	
[X]	Consent Form dated: 8/22/01	AUG 31 2001

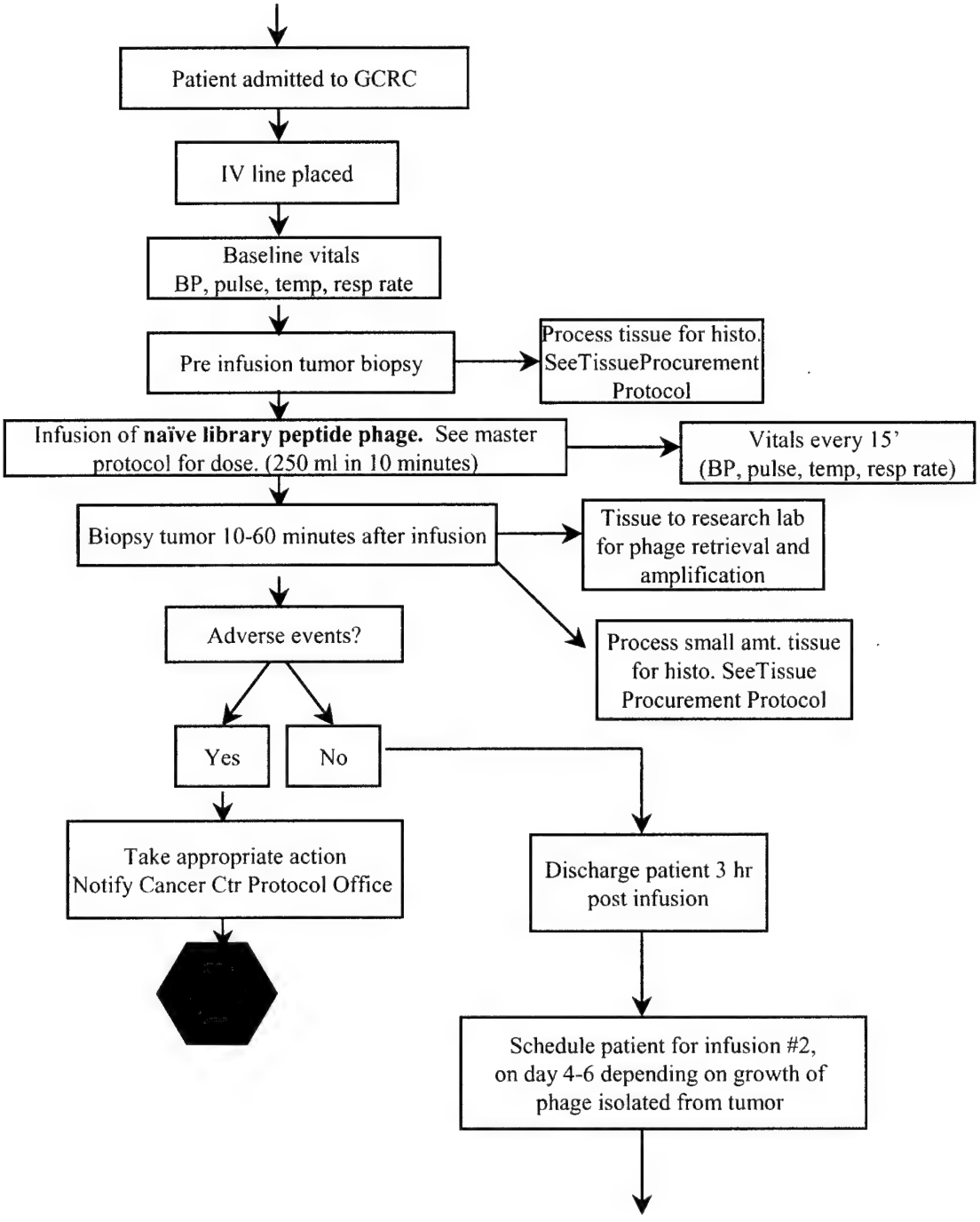
Institutional Signature/Date: 8/31/01

Name and Title of Official: Alan Homans, M.D., Chair, Committee on Human Research in the Medical Sciences

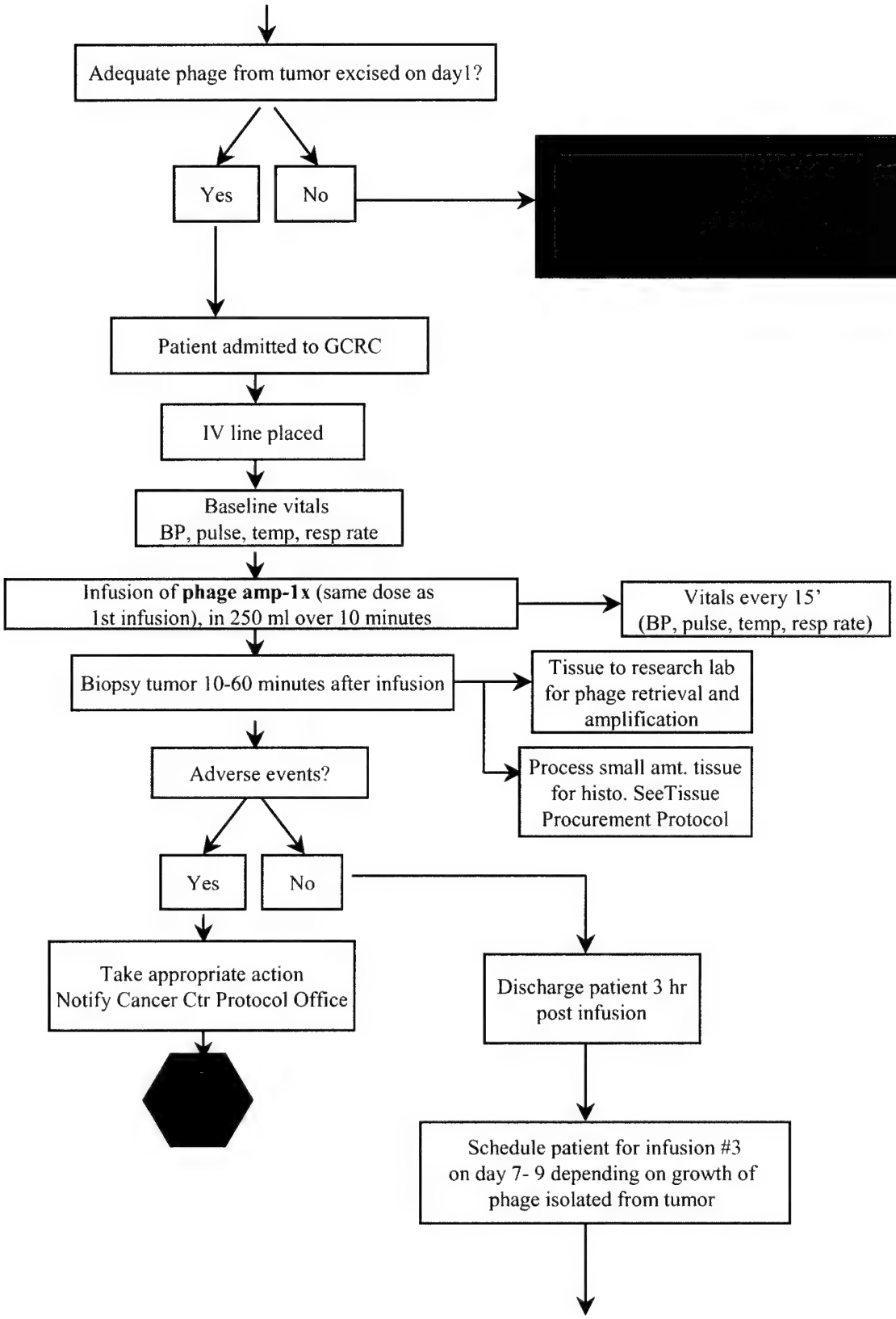
In Vivo Selection of Ligands for Targeted Therapy: Patient Flow Chart



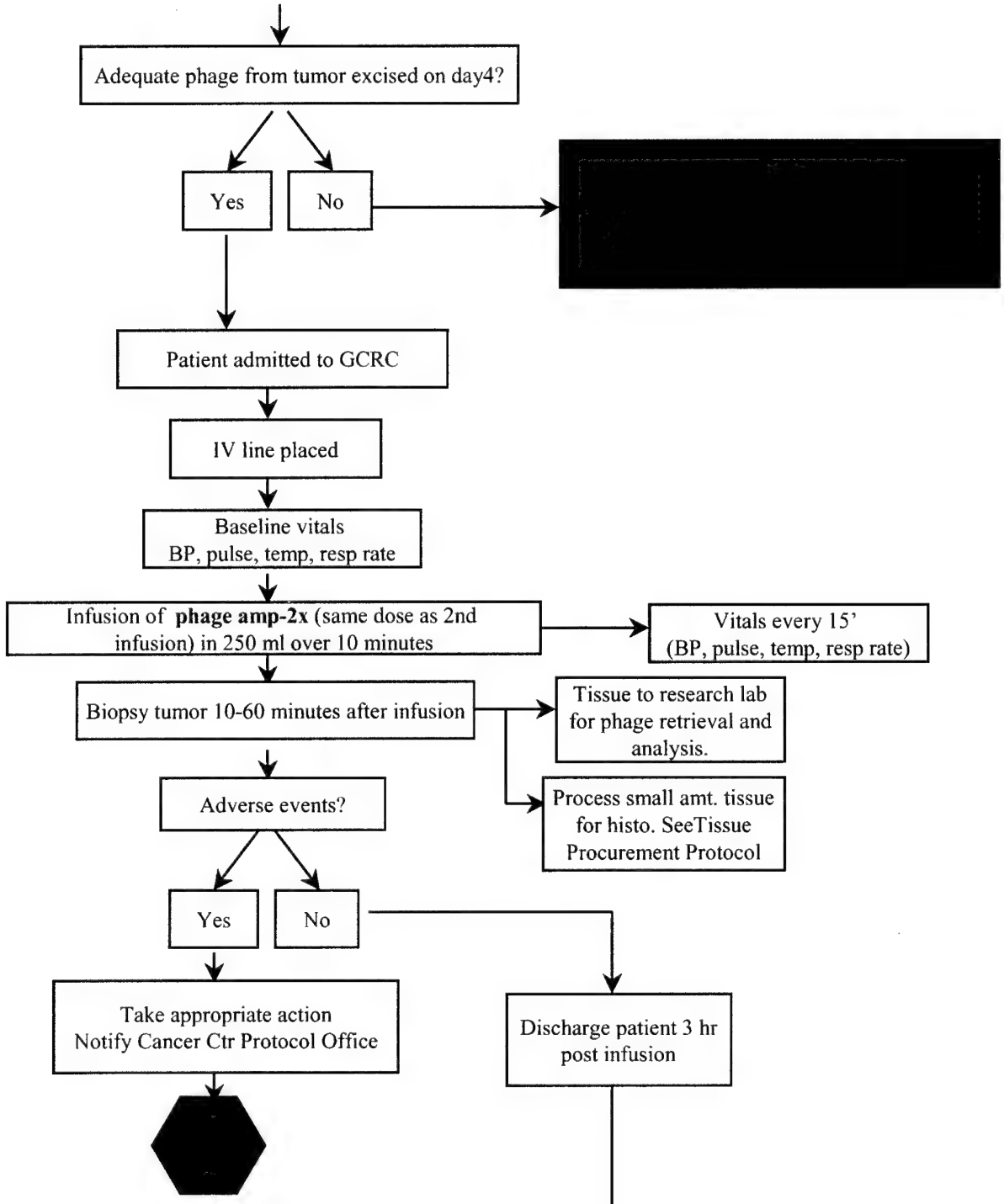
Day 1


Patient admitted to GCRC
IV line placed
Baseline vitals
BP, pulse, temp, resp rate
Pre infusion tumor biopsy
Process tissue for histo. SeeTissueProcurement Protocol
Infusion of naïve library peptide phage. See master protocol for dose. (250 ml in 10 minutes)
Vitals every 15'
(BP, pulse, temp, resp rate)
Biopsy tumor 10-60 minutes after infusion
Tissue to research lab for phage retrieval and amplification
Adverse events?
Process small amt. tissue for histo. SeeTissue Procurement Protocol
Yes
No
Take appropriate action
Notify Cancer Ctr Protocol Office
Discharge patient 3 hr post infusion
Schedule patient for infusion #2, on day 4-6 depending on growth of phage isolated from tumor

Day 4
or
Day 6


Adequate phage from tumor excised on day1?
Yes
No
[redacted]
Patient admitted to GCRC
IV line placed
Baseline vitals
BP, pulse, temp, resp rate
Infusion of **phage amp-1x** (same dose as 1st infusion), in 250 ml over 10 minutes
Vitals every 15'
(BP, pulse, temp, resp rate)
Biopsy tumor 10-60 minutes after infusion
Tissue to research lab for phage retrieval and amplification
Process small amt. tissue for histo. SeeTissue Procurement Protocol
Adverse events?
Yes
No
Take appropriate action
Notify Cancer Ctr Protocol Office
Discharge patient 3 hr post infusion
Schedule patient for infusion #3 on day 7- 9 depending on growth of phage isolated from tumor

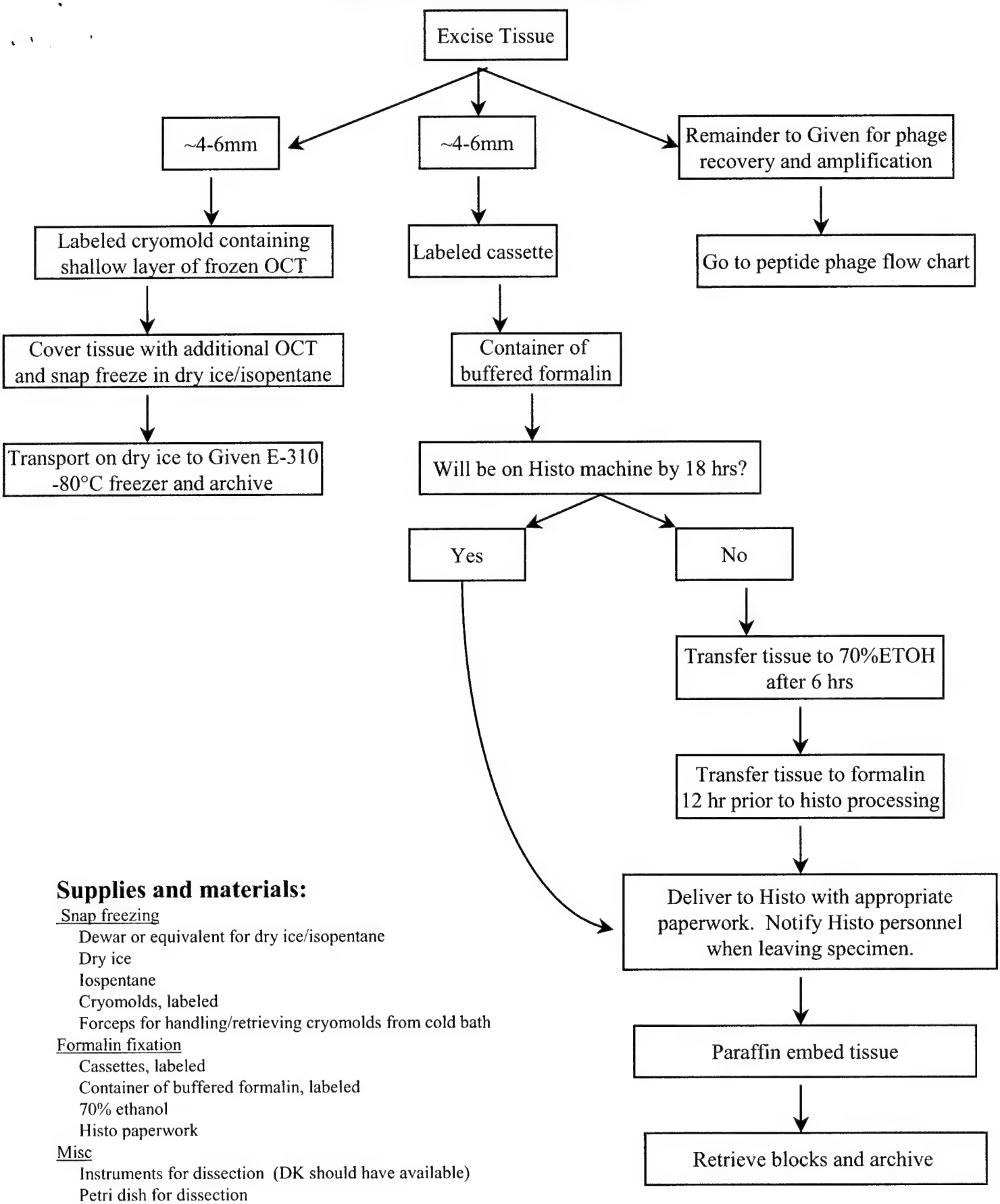
Day 7
or
Day 9



One month after last phage infusion

Six weeks to 2 months after last phage infusion

Tissue Procurement Flow Chart



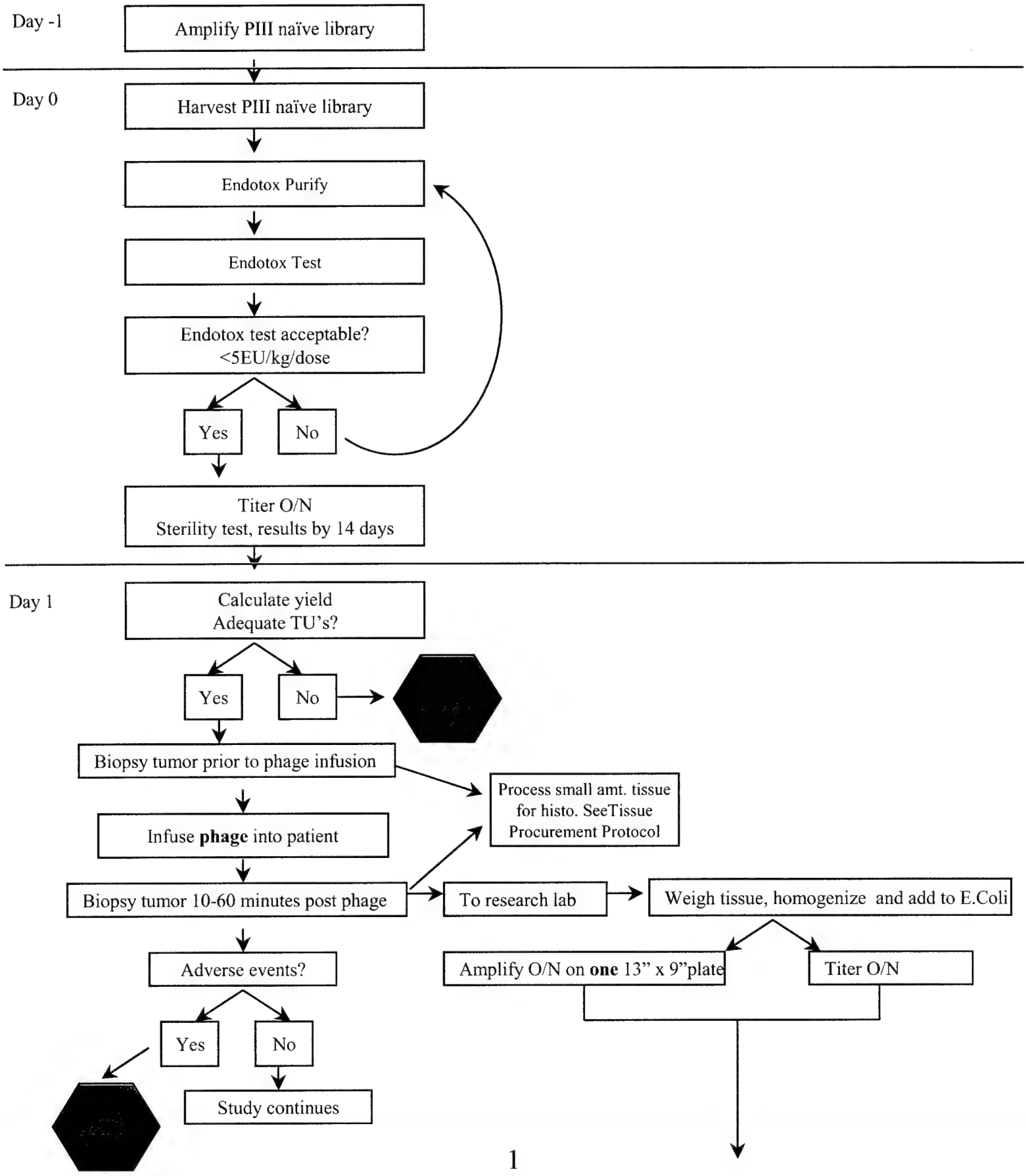
Supplies and materials:

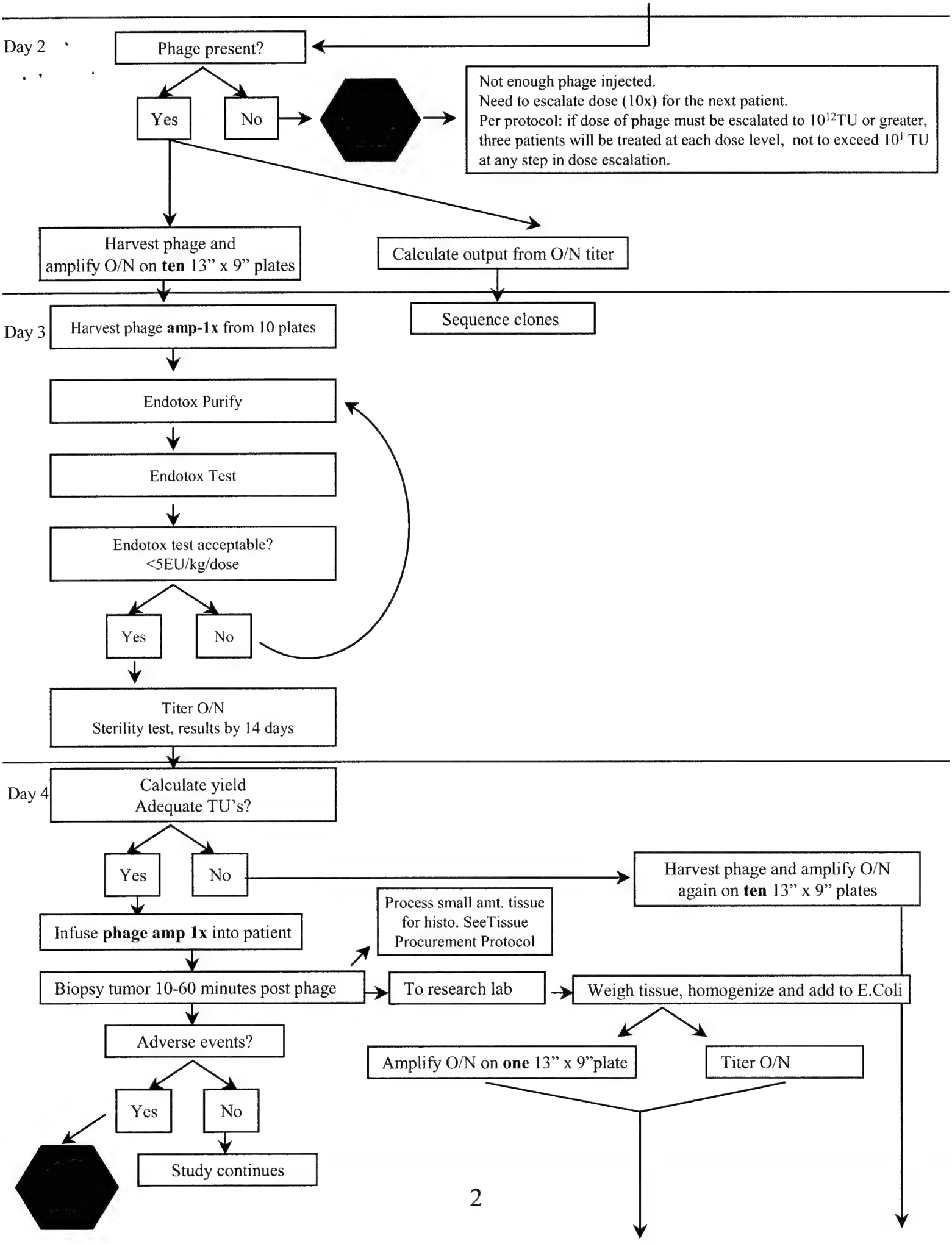
Snap freezing
- Dewar or equivalent for dry ice/isopentane
- Dry ice
- Iospentane
- Cryomolds, labeled
- Forceps for handling/retrieving cryomolds from cold bath

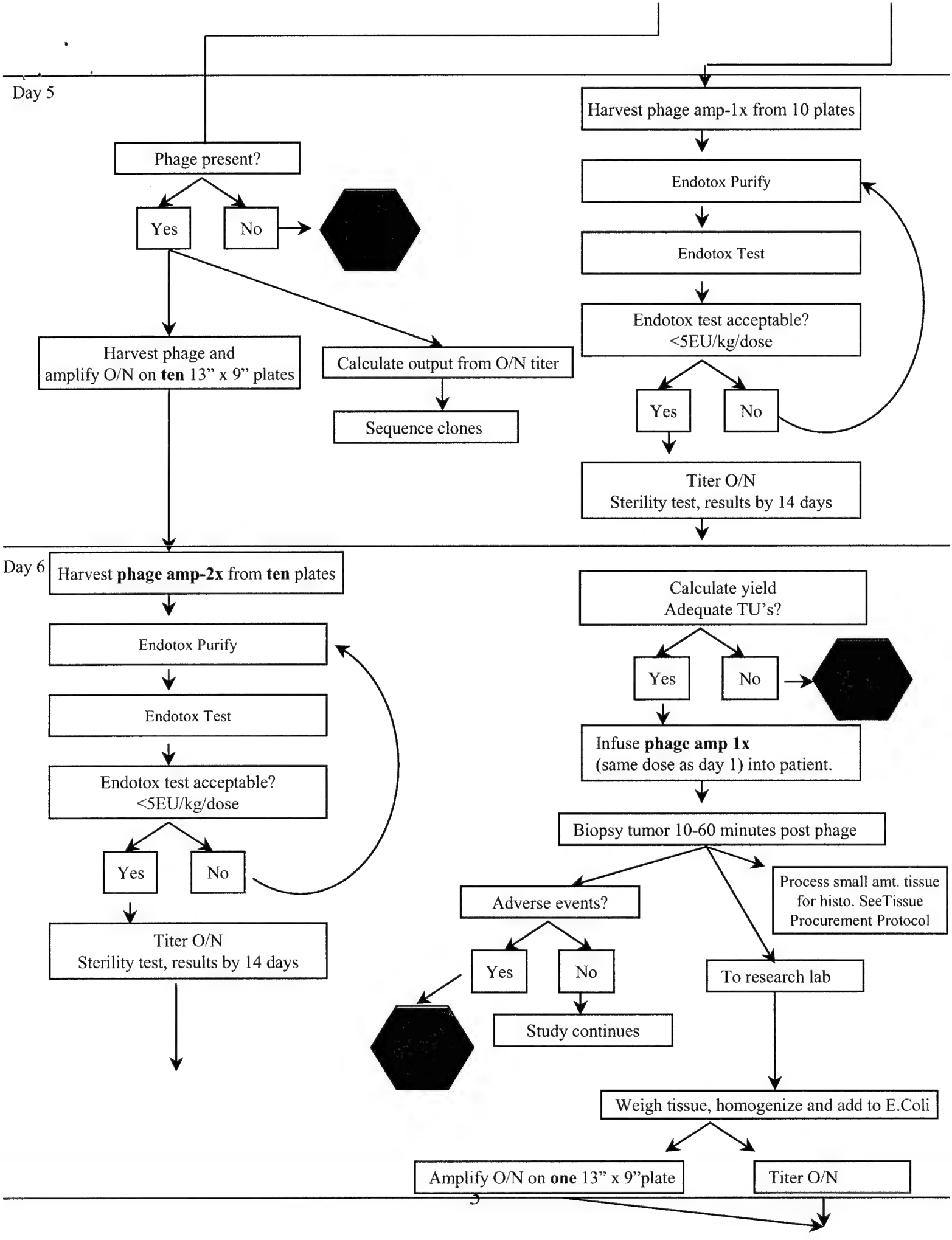
Formalin fixation
- Cassettes, labeled
- Container of buffered formalin, labeled
- 70% ethanol
- Histo paperwork

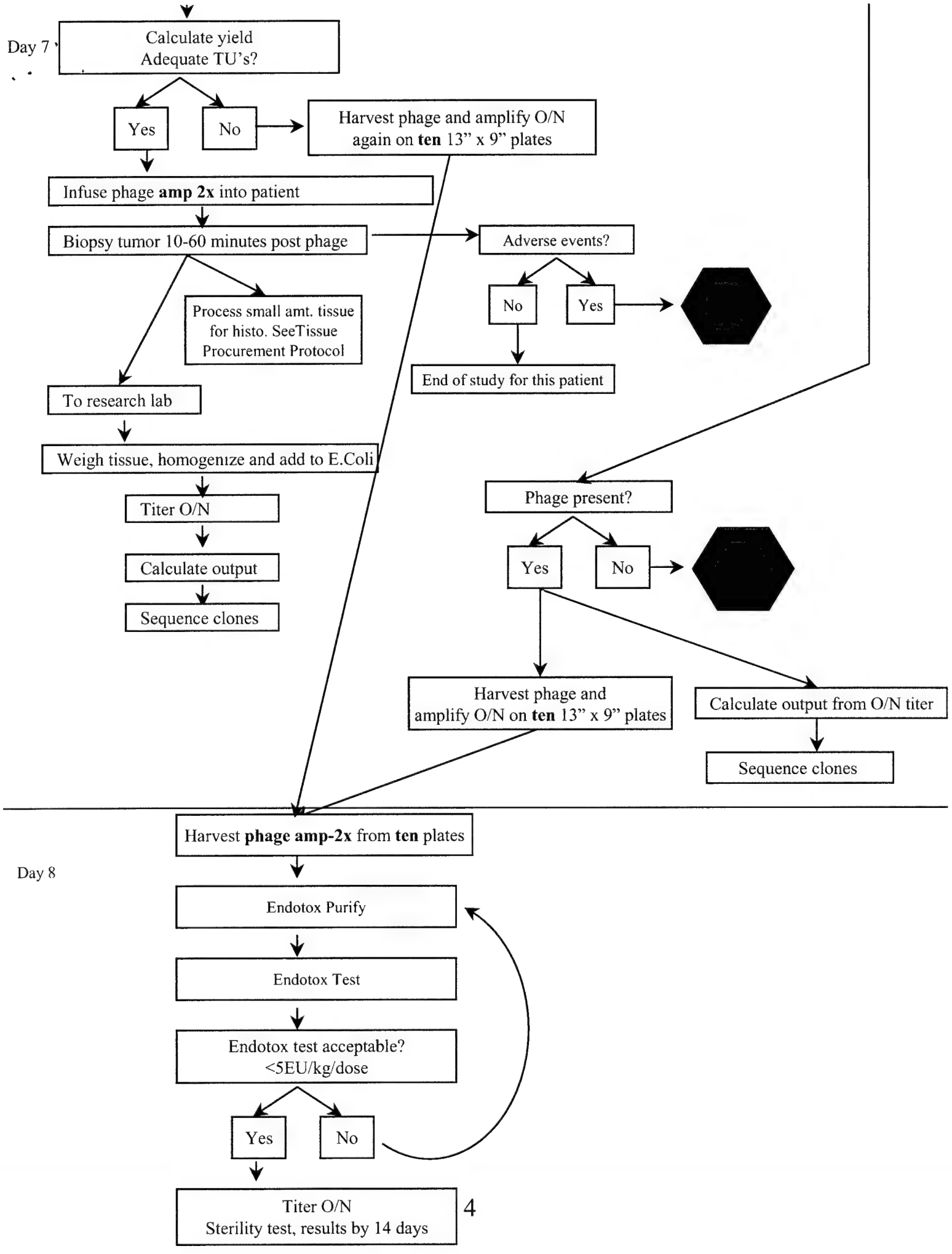
Misc
- Instruments for dissection (DK should have available)
- Petri dish for dissection
- Gloves

In Vivo Selection of Ligands for Targeted Therapy: Research Laboratory Flow Chart for Peptide Phage

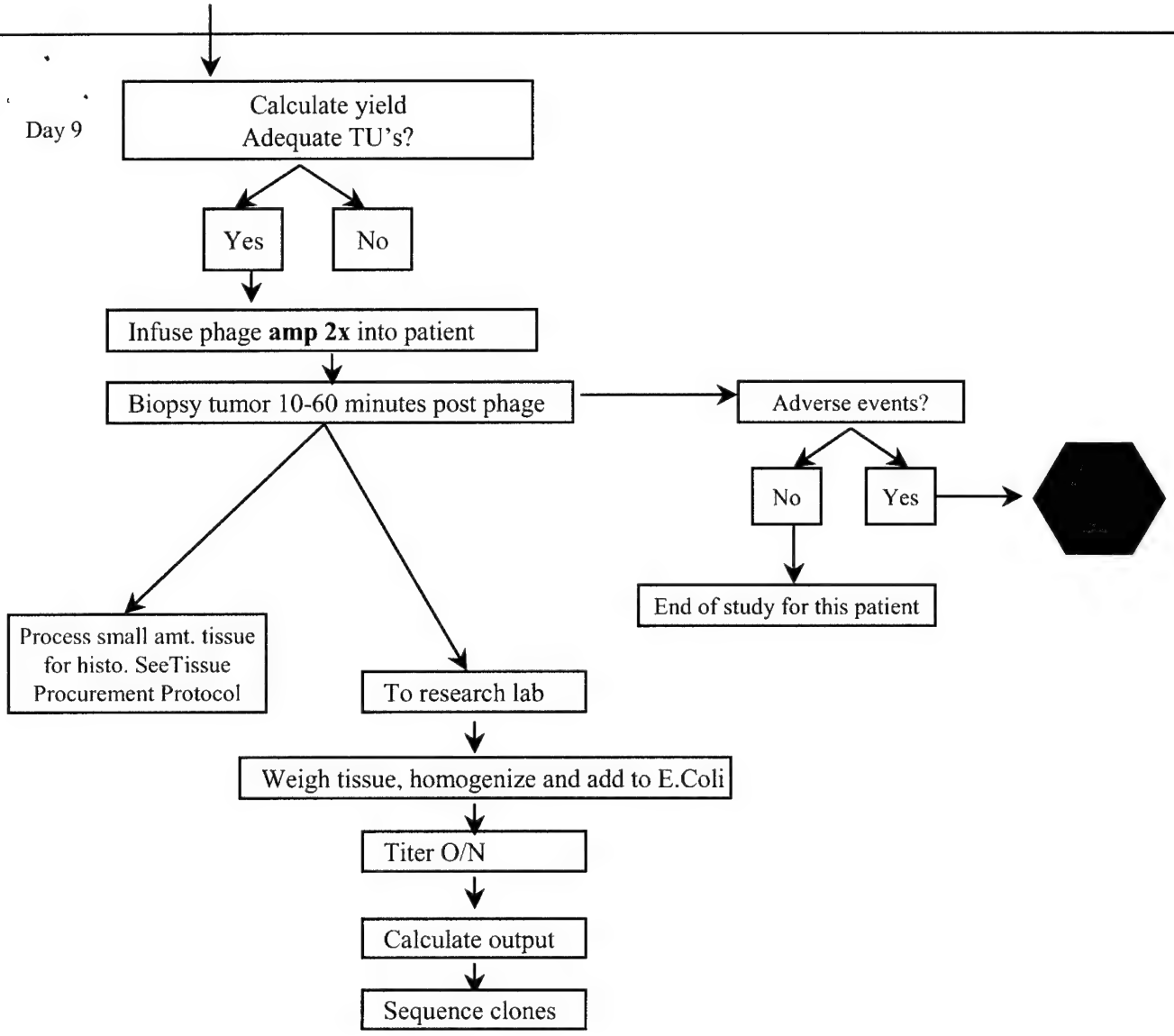



Day 2
Phage present?
Yes
No
Not enough phage injected.
Need to escalate dose (10x) for the next patient.
Per protocol: if dose of phage must be escalated to 10^{12}TU or greater, three patients will be treated at each dose level, not to exceed 10^{1} TU at any step in dose escalation.
Harvest phage and amplify O/N on **ten** 13" x 9" plates
Calculate output from O/N titer
Sequence clones
Day 3
Harvest phage **amp-1x** from 10 plates
Endotox Purify
Endotox Test
Endotox test acceptable? <5EU/kg/dose
Yes
No
Titer O/N
Sterility test, results by 14 days
Day 4
Calculate yield
Adequate TU's?
Yes
No
Harvest phage and amplify O/N again on **ten** 13" x 9" plates
Infuse **phage amp 1x** into patient
Process small amt. tissue for histo. SeeTissue Procurement Protocol
Biopsy tumor 10-60 minutes post phage
To research lab
Weigh tissue, homogenize and add to E.Coli
Adverse events?
Amplify O/N on **one** 13" x 9"plate
Titer O/N
Yes
No
Study continues


Day 5
Phage present?
Yes
No
Harvest phage and amplify O/N on **ten** 13" x 9" plates
Calculate output from O/N titer
Sequence clones
Harvest phage amp-1x from 10 plates
Endotox Purify
Endotox Test
Endotox test acceptable? <5EU/kg/dose
Yes
No
Titer O/N
Sterility test, results by 14 days
Day 6
Harvest **phage amp-2x** from **ten** plates
Endotox Purify
Endotox Test
Endotox test acceptable? <5EU/kg/dose
Yes
No
Titer O/N
Sterility test, results by 14 days
Calculate yield
Adequate TU's?
Yes
No
Infuse **phage amp 1x** (same dose as day 1) into patient.
Biopsy tumor 10-60 minutes post phage
Adverse events?
Yes
No
Study continues
Process small amt. tissue for histo. SeeTissue Procurement Protocol
To research lab
Weigh tissue, homogenize and add to E.Coli
Amplify O/N on **one** 13" x 9"plate
Titer O/N


Day 7
Calculate yield
Adequate TU's?
Yes
No
Harvest phage and amplify O/N again on **ten** 13" x 9" plates
Infuse phage **amp 2x** into patient
Biopsy tumor 10-60 minutes post phage
Adverse events?
No
Yes
End of study for this patient
Process small amt. tissue for histo. SeeTissue Procurement Protocol
To research lab
Weigh tissue, homogenize and add to E.Coli
Titer O/N
Calculate output
Sequence clones
Phage present?
Yes
No
Harvest phage and amplify O/N on **ten** 13" x 9" plates
Calculate output from O/N titer
Sequence clones
Harvest **phage amp-2x** from **ten** plates
Day 8
Endotox Purify
Endotox Test
Endotox test acceptable?
<5EU/kg/dose
Yes
No
Titer O/N
Sterility test, results by 14 days

Day 9


Calculate yield
Adequate TU's?
Yes
No
Infuse phage **amp 2x** into patient
Biopsy tumor 10-60 minutes post phage
Adverse events?
No
Yes
End of study for this patient
Process small amt. tissue for histo. SeeTissue Procurement Protocol
To research lab
Weigh tissue, homogenize and add to E.Coli
Titer O/N
Calculate output
Sequence clones

Lori A. Tull
Consumer Safety Officer
Food and Drug Administration
Center for Biologics Evaluation and Research
HFM-99 Room 200N
1401 Rockville Pike
Rockville MD 20852-1448

Dear Ms. Tull:

Enclosed you will find **BB-IND 9145 with revisions**. We are providing three copies of the entire IND. (You should receive 2 Fed Ex Packages.)

If you have any questions, please contact me at the number listed below or David N. Krag, MD at (802)656-5830.

Revisions are in **bold and underlined**. Attached is a list of the revisions made to the original June 20, 2000 submission of this IND application.

Sincerely,

Mary J. Krupski, CCRP
Clinical Program Coordinator
Vermont Cancer Center
University of Vermont
Given D317
Burlington, VT 05405
Tel (802)656-4270
Fax (802)656-1987
Email: mary.krupski@uvm.edu

Revisions to June 20, 2000 Original Submission of BB-IND 9145

Section 2:

*Page 1, paragraph 3: "250 ml saline" has been changed to **100 – 250 ml saline**; "10 minutes"
has been changed to **approximately 10 minutes**; "at 10 minutes and 24 hours" has been changed to **at 10 - 60 minutes.**

*Page 1, paragraph 4: "within 12 hours" has been changed to **within 12 – 24 hours.**

*Page 1, paragraph 5: "on a large panel of 32 different normal human tissues not from the protocol patient" has been changed to **A panel of different non-tumor tissues will be obtained form the Vermont Cancer Center Tissue Procurement Facility**.

Section 3:

*Page 2, paragraph 6: "250 ml saline" has been changed to **100 – 250 ml saline**; "10 minutes"
has been changed to **approximately 10 minutes**; "at 10 minutes and 24 hours" has been changed to **at 10 - 60 minutes.**

*Page 3, paragraph 1: "within 12 hours" has been changed to **within 12 – 24 hours.**

*Page 3, paragraph 2: "on a large panel of 32 different normal human tissues not from the protocol patient" has been changed to **A panel of different non-tumor tissues will be obtained form the Vermont Cancer Center Tissue Procurement Facility**.

Section 4:

Both clinical protocols have been revised according to suggestions made by the UVM Committee on Human Subjects, the UVM Protocol Review Committee, and the Department of Defense Committee on Human Subjects.

Section 5:

*Page 2, 3rd paragraph has been changed to read: "The peptide-phage that we will be using for Phase I clinical trials will be nearly identical to that used in our animal studies. **Peptide-phage for clinical use will be produced either without any protease inhibitors, or with Trasylol--a drug already approved for human use in treating pancreatitis."**

*Page 6: Stability of substance has been changed to read: "Phage particles are stable and infective for **at least 3** years when stored at 4°C. However the stability of the peptides displayed on the phage particles **is** unknown and is likely to vary from peptide to peptide. (We have preliminary data on one peptide showing stability to 7 weeks.) The substance will be stored at 4°C prior to use in patients and will be **used within approximately 72 hours.**

*Page 6: Drug Product: Components used in manufacture section: "**not for human use. May replace with Trasylol for clinical studies."** has been added to the EPI line.

*Page 6: Drug Product: Components used in manufacture section: "**not for human use. "** has been added to the PPI line.

*Page 6: Drug Product: Components used in manufacture section: **Sigma Product Number: X-114** has been added to the Triton X-114 line.

*Page 6: Product Preparation, sentence 5: the phrase "and the phage suspension is passed through a 0.45 μm cellulose acetate filter" has been deleted.

*Page 7, 1st paragraph: the phrase "0.45 μm cellulose acetate filter, followed by passage through a" has been deleted. "**Please note that future preparations will employ a low protein binding 0.2 μm polyethersulfone (PES) filter unit"** and "**As stated earlier (Section 5- page 2), peptide-phage for clinical use will be produced either without any protease inhibitors, or with Trasylol--a drug already approved for human use in treating pancreatitis"** have been added to the end of the paragraph.

*Page 7, paragraph 6, last sentence (Therefore, to perform three screens…) has been replaced with: "**Sterility testing will be done for each preparation of peptide phage that is injected. The results will not be available to us prior to administration of the product. However, in the event of a test failure, appropriate actions will be taken as outlined in the clinical protocol.**"

*Page 8, paragraph 1 (Endotoxin Testing), sentence 4 has been changed to read: "**We have been advised by the FDA that** the maximum amount of endotoxin allowed in a substance that will be injected IV into humans is **5** endotoxin units (EU) per kg of patient weight per **dose**.

*Page 9, top of the page has been revised to read: "If FDA endotoxin limit=**5** EU/kg/**dose**, then the limit for an average 70 kg individual = **350** EU/dose

* Product was positive for endotoxin between 1:20 and 1:40 dilution, or 10-20 EU/ml (based on 0.5 EU/ml positive standard). **The final volume of the phage preparation from 4/3/00 was 2.5 ml. Therefore the preparation contained 25-50 EU**, well **below** FDA limits, even for lighter weight patients.

Section 6:

*Page 3, 2nd paragraph: "IV injection" has been changed to "**a single** IV injection."

*Page 3, 4th paragraph: The following has been added at the end of the paragraph: "**As in Study IV, these studies were designed to test whether sequential injection of peptide-phage amplified from tumor tissue is toxic. However, this study circumvents the complication of technically difficult mouse survival surgeries and tests the peptide-phage preparations directly.**"

*Page4, 2nd paragraph: The last sentence has been changed to read: "Therefore, it was **difficult to predict which mice were most likely to remain alive for the entire duration of the study, as many died even with no treatment. Study mice needed to have at least three** tumors for our studies, yet, ideally, were healthy enough to survive 3 surgeries **within 5 days,** and live to the 3-week endpoint (as in Study IV).

*Page 4, paragraph 3 has been added and reads: "**Technical difficulties were encountered while injecting peptide phage into the tail veins of the mice. All mice received the full dose planned. In a minority of mice some of the dose went into subcutaneous tissue in addition to the intravenous dose. This appeared due to the small size of the tail vein and the viscosity of the fluid injected. (Please refer to the table in the Appendix entitled**

Phage Input Summary of Mice Injected for Toxicity Studies.)" A copy of this table is attached.

*Page 4, 4th paragraph: First sentence: 31 has been corrected to read "**32**." The second sentence has been changed to read: "**The mouse that died before the study endpoint was one of the** three surgical mice (#3), **which** died while under general anesthesia following the removal of tumor during the second surgery. Fourth sentence: 32 has been corrected to read "**33**."

*Page 4, 5th paragraph: The phrase "**except for the one that died in the restraint**" has been removed from the first sentence. The sentence now reads: "All non-surgical mice (n=28) appeared normal for the study duration as observed by activity level, appearance of coat, and posture."

*Page 5, 1st paragraph: The last sentence has been changed to read: "For example, **s**urgical **m**ouse #2, **in Study IV**, weighed 47.6 g on the first day of surgery, and had 3 tumors removed in 5 days weighing 600 mg, 403.6 mg, and 431.8 mg respectively."

*Page 5: The 3rd paragraph has been changed to read: "**At** three days **most** blood and tissues were **highly** positive for infective **phage** . **At t**hree weeks following phage injection, regardless of preparation (naïve, φ Amp1x, or φ Amp2x) all tissues were negative for infective phage in all mice **except one: surgical mouse #2. Surgical mouse #2 had some phage detected in most tissues at three weeks, although much less than the number typically detected in tissues three days after injection. The only tissue that did not have infective phage detected at three weeks in this mouse, interestingly, was tumor tissue."**

*Page 5: The lastparagraph has been deleted.

*Page 6: The Summary of Phage Titer Results table has been revised to show numbers of mice in each group, ie **(n=x)**. Study II has been divided to reflect **ϕamp 1x** and **ϕamp 2x** injected mice. Footnote 3 has been changed to read: "All tissues positive **for phage** except: mouse #3 blood and liver, and mouse #2 spleen."

*Page 7, Summary of IHC Results table: N=2 has been changed to **n=1** for 3 week mouse in Study IV. Results for this have been changed from "-/+" to "-." Footnote 6 has been deleted.

*Page 8: Sentence 4 has been changed to read "**BalbCs also had a strain-specific steatosis of the liver**."

*Page 8, Summary of H&E Results: "**1 liver with lymphoid aggregates**" has been added to the 3 Week Harvest results for Study II ϕamp2x mice. Study I: n=5 has been corrected to read "**n=4**" for the 3 Day and 3 Week results in Study I.

*Page 23, Weights section: Changed to read "The mice injected with φ Amp1x dropped an average of 9.1% (n=7) of their body weight on day 1 following injection but **their weights** had returned to baseline by day 2. The mice injected with φ Amp2x either maintained or gained weight throughout the remainder of the study.

*Page 23, Phage Titers section: Changed to read "Three days after phage injection, there were infective phage present in all of the tissues except for the blood and liver of mouse #3 and the spleen of mouse #2. **(Both mouse #2 and mouse #3 were injected with** φ Amp1x.) No infective phage were detected in any of the tissues collected three weeks after injection of either φ Amp1x or φ Amp2x.

*Page 25, Study II-Graph of Body Weights After Injection of ϕAmp2x: Legend for the graph corrected for mouse #.

*Page 27, Study II-Table of Body Weights After Injection of ϕAmp2x: Mouse ID numbers corrected.

*Page 28, Phage Titer Results: Study II section: Bottom half of table corrected to show ϕAmp**2**x Injection, instead of ϕAmp1x, at 3 Days and 3 Weeks.

*Page 29, IHC Results: Study II section: Bottom half of table corrected to show ϕAmp**2**x Injection, instead of ϕAmp1x, at 3 Days and 3 Weeks.

*Page 34, H&E Results: Study II section: Top half of table, mouse 1 results corrected to read **NA** for each organ. Bottom half of table corrected to show ϕAmp**2**x Injection, instead of ϕAmp1x, at 3 Days and 3 Weeks. Bottom of page: footnote 7 added that reads "**not available, mouse died in restraint prior to injection**."

*Page 40, Methods: Study III:

Line 1: 1.5×10^{10} has been corrected to read **3.8×10^{9} TU's.**

Line 3: 1.4×10^{10} has been corrected to read **3.6×10^{9} TU's**.

Line 6: 1.4×10^{10} has been corrected to read **2.8×10^{9} TU's**.

*Page 53, Number of Peptide-Phage (TU) Injected: Study IV

Line 4: 6.4×10^{9} has been corrected to read **6.4×10^{8}**.

Line 4: 9.3×10^{11} has been corrected to read **8.2×10^{10}**.

Section 8:

*Appendix Table of Contents:

3. "**Tumor Homogenization for Phage Titering and Amplification Protocol"** has been added to the list of protocols.

"**4. Phage Input Summary of Mice Injected for Toxicity Studies**" has been added.

"**7. Certificates of Analysis**" has been added.

*Phage Amplification and Harvesting Protocol, Day 1, 2nd line has been changed to "Prepare **10** pans 2xYT KanTet."

*Phage Amplification and Harvesting Protocol, Day 2, line 7 has been changed to "Check OD_{600} frequently; do not exceed OD_{600}=0.**3**"

*Phage Amplification and Harvesting Protocol, Day 2, line 8 has been changed to "At OD_{600}=0.**3** shake at 70rpm, 10min."

*Phage Amplification and Harvesting Protocol, Day 2, Infect library, line 9 has been changed to "Resuspend and pool cells in a total of **10**ml 2xYTKanTet media."

Phage Amplification and Harvesting Protocol, Day 2, Infect library, line 10: a "" has been added at the end of the line.

*Phage Amplification and Harvesting Protocol, Day 2, Infect library, line 11 has been changed to "Spread 1ml to each of **ten** pans under hood."

Phage Amplification and Harvesting Protocol, Day 3, line 3: a "" has been added to the end of the line.

Phage Amplification and Harvesting Protocol, Day 3, line 11: a "" has been added to the end of the line.

*Phage Amplification and Harvesting Protocol, Day 3, line12, "Filter through 0.45 μm CA syringe filters into two 15 cc orange-capped centrifuge tubes" has been deleted.

*Phage Amplification and Harvesting Protocol, "***Do not use protease inhibitors if preparing phage for human use. May replace with Trasylol for human use**" has been added at the bottom of the page.

Endotoxin Purification and Removal Prior to In Vivo Phage Injection protocol: a "" has been used as a footnote marker whereever EPI's or PPI's are mentioned. Step 18 "Filter supernatant through a 0.45 μm cellulose acetate membrane…" has been deleted. "**Do not use these protease inhibitors if preparing phage for human use. May replace with Trasylol for human use"** and "****This filter will be replaced with a Millipore 0.2μm PES syringe filter in future preparations."**have been added at the bottom of the page.

*Phage Titering Protocol for In Vivo Screenings, line #2, the mispelled word "bottow" hasbeen changed to "bottom."

*The protocol entitled "**Tumor Homogenization for Phage Titering and Amplification Protocol**" has been inserted.

*Reagents used in peptide-phage production, EPI (eukaryotic protease inhibitors): the following has been added: "**Not for human use. May replace with Trasylol for human use.**"

*Reagents used in peptide-phage production, PPI (prokaryotic protease inhibitors): the following has been added: "**Not for human use."**

*Phage Titering Protocol for Harvested Organs, "*** These inhibitors are not to be used if phage from titering will be harvested and amplfied for use in human studies. Trasylol may be used as a replacement"** has been added to the bottom of the page.

*A table entitled "**Phage Input Summary of Mice Injected for Toxicity Studies**" has been added.

Phage Input Summary of Mice Injected for Toxicity Studies

Study	Strain	ID	Phage Injected	Date Injected	Volume Injected	TU/injection	Estimated % of total in vein*
I	FVB	1	naïve	08/09/2000	100 µl	Not available	100
		2			100 µl		100
		6			100 µl		100
		7			100 µl		100
	BalbC	4			100 µl		100
		5			100 µl		100
		9			100 µl		100
		10			100 µl		100
II	MRL	8285.1	ϕamp1x	02/02/2000	250 µl	6.4×10^8	100
		8285.2			250 µl		50
		8285.3			250 µl		100
		8285.4			250 µl		100
	FVB	8192.1			250 µl		100
		8192.2			250 µl		100
		8192.3			250 µl		100
	MRL	8285.5	ϕamp2x	02/04/2000	245 µl	8.2×10^{10}	100
		8285.6			245 µl		100
		8285.7			245 µl		100
		8285.8			245 µl		100
	FVB	8192.4			245 µl		100
		8192.5			245 µl		100
		7678.1			245 µl		100
		7678.2			245 µl		100
III	MRL	8680.1	naïve	04/03/2000	250 µl	3.8×10^9	75
		8680.2			250 µl		40
		8680.3			250 µl		40
		8680.4			250 µl		50
		8680.5			250 µl		<10
		8680.6			250 µl		100

* Please refer to Pharmacology and Toxicology (p. 4) for discussion.

Study	Strain	ID	Phage Injected	Date Injected	Volume Injected	TU/injection	Estimated % of total in vein*
III	MRL	8680.1	ϕamp1x	04/05/2000	250 µl	3.6×10^9	90
		8680.2	↓	↓	250 µl	↓	80
		8680.3			250 µl		100
		8680.4			250 µl		90
		8680.5			250 µl		95
		8680.6			250 µl		25
		8680.1	ϕamp2x	04/07/2000	200 µl	2.8×10^9	90
		8680.2	↓	↓	200 µl	↓	20-30
		8680.3			200 µl		25
		8680.4			200 µl		100
		8680.5			200 µl		40-50
		8680.6	↓	↓	200 µl	↓	75
IV	MRL	7593.1	naïve	09/21/1999	NA	Not available	NA
			ϕamp1x	09/22/1999	↓	↓	↓
			ϕamp2x	09/24/1999			
			ϕamp3x	10/15/1999	100 µl	↓	100
	MRL	7834	naïve	01/31/2000	260 µl	2.6×10^9	90
			ϕamp1x	02/02/2000	250 µl	6.4×10^8	100
			ϕamp2x	02/02/2000	245 µl	8.2×10^{10}	100
	MRL	7834.1	naïve	04/03/2000	250 µl	3.8×10^9	90
			ϕamp1x	04/05/2000	250 µl	3.6×10^9	100

NA=not available

* Please refer to Pharmacology and Toxicology (p. 4) for discussion.